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Mineralization of inositol-bound phosphorus in soil

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NOTE TO USERS

MINERALIZATION OF INOSITOL-BOUND PHOSPHORUS IN SOIL

by

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INTRODUCTION.

The name "phytin" was coined to designate the mixed calcium-magnesium salt of inositol hexaphosphoric acid occurring naturally in plants, especially in the seeds. Recently, strong circumstantial evidence has been accumulated that soils contain inositol hexaphosphate together with lower phosphates produced by partial hydrolysis of the hexaphosphate and that the phosphorus so bound may constitute a considerable fraction of the organic phosphorus of the soil.

The origin of this material is in doubt. It has been assumed that plant residues are the source and that the large amount of inositol-bound phosphorus in the soil is the result of a long accumulation process made possible by its great stability under conditions existing in the soil. However, recent work has shown that there is very little or no phytin in the vegetative parts of plants, and that practically all the phytin phosphorus in the seed is mineralized during germination. Furthermore, most plant tissues possess the hydrolyzing enzyme phytase, which presumably can hydrolyze an appreciable part of the phytin before the latter can react with the soil. As yet there has been no critical demonstration

enzymes are ~~a~~ reversible; hence, synthesis might occur.

that micro-organisms can synthesize phytin or inositol phosphoric esters.

The importance of soil phytin as a source of phosphorus for plants is also in doubt. Solution culture experiments have shown that plants can effectively utilize phytin as such, but responses to phytin added to soils have been positive only under neutral or alkaline conditions. Under acid conditions (about pH 5) there has usually been no response at all. This lack of response has been attributed to the great insolubility of aluminum and iron phytates under acid conditions, and it has been assumed that this insolubility prevents both the direct absorption of phytin by plants and the mineralization of the phytin phosphorus.

While the above solubility explanation for the differing availability of phytin phosphorus may well be correct, there is a possible alternative. Various micro-organisms found in the soil have been shown to possess phytase activity. Since micro-organisms permeate the soil more thoroughly than do plant roots, it is possible that mineralization of phytin phosphorus, under the influence of microbial phytase, is quantitatively of more importance than is the absorption of phytin as such by plants. The positive responses of plants may be due to the inorganic phosphorus so produced. Since the extent of this mineralization will depend both on the solubility of the phytates and on the level of phytase

activity, the lack of response at low pH's may be due to a low enzyme activity and the positive responses at higher pH's to a higher activity.

The present work is a study of the solubilities of some of the salts of inositol phosphoric acids under varying conditions, the source and some of the characteristics of phytase in soils, and the relative importance of solubility and phytase activity in controlling the mineralization of inositol-bound phosphorus in soil. The object of this work was to obtain a clearer appreciation of the importance of phytin as a source of phosphorus for plants.

REVIEW OF LITERATURE.

Phytin appears to be universally present in the seeds of plants. Rose (60), writing in 1912, states that up to that time no case had been reported of a seed not containing this compound. The phosphorus so combined may constitute from 20 to 90 per cent of the total phosphorus in the seed and is thus an important phosphorus fraction.

Studies of this material have been made mainly by workers interested in animal and human nutrition, more especially where cereals and grains are the main constituents of the diet. Such diets tend to produce rickets, and it has been postulated that the causative factor is the phytin, which limits the absorption of calcium due to the precipitation of insoluble calcium phytate in the intestines. The factors investigated have been the solubility of different salts and the hydrogen-ion concentration, both as affecting the solubility and as catalyzing hydrolysis, and the characteristics of the hydrolyzing enzyme phytase. In soils, the main lines of work have involved isolation and characterization of the materials in soil extracts, fixation by soil, mineralization of the phosphorus, and the response of plants

to soil additions.

Solubility

Inositol hexaphosphoric acid, or phytic acid, forms acid, neutral, or alkaline salts depending on the nature and amount of base present and the precipitating medium. The solubility of the calcium salt has been investigated by Hoff-Jørgensen (32), who precipitated the salt at various pH values, the concentration of the phytic acid being about 0.02 M and the calcium about 0.1 M. Precipitation began at about pH 2.7 but was apparently not complete even at pH 7.6. The precipitate increased in calcium content as the pH was raised until, over the range from pH 4.6 to 6.7, the precipitate was the penta-calcium salt. Above pH 6.7 the proportion of calcium increased. X-ray analysis showed that the salt precipitated was fully amorphous. The solubility greatly decreased with increase of temperature and also with decreasing ionic strength of the solution. Calculated solubility products for the penta-calcium salt at 37° C, ranged from $10^{-18.5}$ (pH 4.2) to $10^{-20.5}$ (pH 7.6) in 1 M sodium chloride, and from $10^{-21.7}$ (pH 4.2) to $10^{-25.2}$ (pH 7.4) in 0.2 M sodium chloride. Comparative solubility studies showed that over the range pH 4.5 to 7.5 secondary calcium phosphate ($CaHPO_4$) was more soluble than penta-calcium phytate and hydroxy apatite, with the

phytate somewhat more soluble than the hydroxy apatite. McCance and Widdowson (47) found that the calcium salt was soluble at pH 2.8, but began to precipitate at pH 3. The magnesium salt was more soluble than the calcium salt between pH 6.0 and pH 7.0 and even more so as the pH fell, being fully soluble below pH 5.

The solubility of iron phytate has not been thoroughly investigated. McCance et al (46) mixed solutions of ferrous and ferric ammonium sulfate with sodium phytate and disodium phosphate, and found that at pH 6.5 the ferrous and ferric phytates were less soluble than the corresponding phosphates or hydroxides. The precipitation of ferric phytate under acid conditions is the basis for most methods of determining phytin. Heubner and Stadler (31) precipitated phytin in 0.6 per cent hydrochloric acid using ferric chloride also in 0.6 per cent hydrochloric acid, and most subsequent methods have been refinements of this. Earley (19) has found that until about 3.5 times the theoretical amount of iron has been added the reaction is non-stoichiometric, but that at this excess or greater the precipitate is the tetraferric salt.

Hydrolysis

Phytase absent.

Soluble phytates are relatively stable toward acid or

alkaline hydrolysis, especially the latter. Plimmer and Page (55) found that they could determine inorganic phosphorus, in the presence of phytin, by precipitation with ammonium molybdate in N/2 to N nitric acid. During the 48 hours required to form the ammonium molybdiphosphate precipitate, no decomposition of the phytin occurred and the determination of the inorganic phosphorus was quantitative. Fleury (22) investigated the hydrolysis of sodium phytate by heating it at 120° C for 8 hours at pH values ranging from 0 to 14 and also with the acidity increased up to 10 N with sulfuric acid. Hydrolysis was complete at pH 3 but rapidly decreased at higher pH values, being 50 per cent at pH 6 and nil at pH 14. In 5 N acid there was a minimum of 50 per cent hydrolysis which increased to 90 per cent in 10 N acid. Plimmer (54) heated phytin in N sodium hydroxide for 32 days at 75° C and found no hydrolysis. Wrenshall and Dyer (72) boiled phytin in the presence of sodium hypobromite and found very little decomposition of the phytin. Under the same conditions soil organic matter was fully decolorized.

Phytin is relatively stable to heat. Widdowson (68) made pastry with flour containing active phytase and found that after cooking there had been little destruction of the phytin. The quick moist heating inactivated the phytase but had little effect on the phytin.

Phytase present.

If phytase is present the hydrolysis of phytin can be both rapid and extensive. Hoff-Jørgensen and Porsdal (33) state that with dilute solutions and under optimum conditions hydrolysis is completed in 10 to 15 minutes.

It is appropriate at this stage to consider the sources and characteristics of phytase. Phytase activity has been found in some fungi by Dox and Golden (18) and by Kawahara (37), in the intestinal lining of rats by Patwardhan (51) and in the erythrocytes and blood plasma of some lower vertebrates especially birds by Rapoport and Guest (57). Berthelot (6) found that yeasts, bacteria, fungi and algae could readily utilize phytin, thus indicating the presence of phytase in these organisms. Giri (26) determined the phytin and the relative phytase activity in many grains, roots and green vegetables. Phytase activity was found in all cases and in general the greater the amount of phytin present the lower was the phytase activity. As yet there has been no work on the sources or amounts of phytase in soils, although the presence of phytase in soils may be inferred from the work of Pearson, Norman and Ho (52).

Critical values for reaction and temperature are variously quoted and some representative figures are listed below.

Source	pH			Temp. ° C			Authority
	Min.	Opt.	Max.	Min.	Opt.	Max.	
Rye	-	5.4-5.8	-	-	55	-	Hoff-Jorgensen (33)
Wheat	-	-	-	-	55	-	Collatz et al. (12)
Wheat	3	5.5	7.3	-	-	-	Kolobkova (39)
Barley	-	4.7-5.4	-	-	-	-	Minkovska (49)
Oats	-	4.9-5.7	-	-	-	-	Lindenbaum (41)
Rice bran	-	4.3	-	-	-	-	Horiuchi (34)
Peanuts	1.7	4.8-6.0	8.9	-	-	-	Fontaine et al. (23)
Cotton seed	-	4.8	-	-	-	-	Fontaine et al. (23)
Aspergillus	-	4.67	-	-	50	-	Kawahara (37)
Malt	-	5.2	-	-	-	50	Luers and Silbereisen (44)
Rat intestine	-	7.8	-	-	-	-	Patwardhan (51)
Avian plasma	-	5.7-7.2	-	-	-	-	Rapoport et al. (57)

These varying values may well be due to the varying sources of the enzyme but are also probably due in part to the differing experimental conditions under which they were determined. The enzyme appears to be relatively stable, as are most of the phosphatases. Boutwell (9) heated dry wheat bran at 125 to 130° C for 10 hours and found that the phytase activity was not much reduced.

There has been little critical work on the hydrolysis of phytin by phytase, although many scattered observations have been made. The work of Hoff-Jørgensen and Porsdal (33) is the most extensive. Working with rye-bran phytase they found that various hydroxy organic acids increased the activity of the phytase, irrespective of any action they might have on the substrate, and that with increasing amounts of these acids

up to 1 per cent the increased activity was accompanied by a decrease in optimum pH from about 5.6 to 5.1. Lactic and citric acids were particularly effective. They also found an interaction between temperature and optimum pH. At 55° C the optimum pH was about 4.9, while at 20° C the optimum pH was 5.4.

Under conditions normally found in biological systems dissolved phytin is relatively stable towards chemical hydrolysis but is not stable towards phytase induced hydrolysis. The degree of hydrolysis appears to depend on the solubility of the phytin. Wrenshall and Dyer (72) found that bran phytase would not hydrolyze suspensions of iron and aluminum phytate when the pH was about 5. Kaila (36) added calcium and aluminum to sodium phytate and adjusted the reaction within the range of pH 8 to 9. After incubation with bran phytase for 90 days he found that the hydrolysis of the sodium phytate was 96 per cent while with calcium present it was 68 per cent and with aluminum present it was 15 per cent.

Inositol-bound Phosphorus in Soils

That phytin is present in soil has not as yet been proved by classical chemical methods, but there is a fairly large body of indirect evidence which well supports the

conclusion that it does exist in the soil. Yoshida (73) was able to isolate from an Hawaiian soil an alcohol-insoluble material which, after acid hydrolysis, gave inositol. Wrenshall and Dyer (72), extracted soil with sodium hydroxide and after destroying the organic matter by hypobromite oxidation, were able to isolate a material by an iron precipitation. They removed the iron from this precipitate with sodium hydroxide and then titrated the filtrate with ferric chloride according to the method of Heubner and Stadler (31). The resulting precipitate had an iron/phosphorus ratio of 1.23 as compared to the 1.19 factor used by Heubner and Stadler. The sodium hydroxide solution of this salt gave a positive Fischler and Kürten test (21). Bower (10), working with Gray Brown Podzolic, Prairie and Wiesenboden soils and following a method similar to that of Wrenshall and Dyer, obtained, under acid conditions, an iron precipitate which he analyzed for phosphorus and inositol. The resulting inositol/phosphorus ratio was found to agree fairly well with that of inositol hexaphosphoric acid. The filtrate from the iron precipitation together with the acid washings from that precipitate, when treated with calcium hydroxide under alkaline conditions, gave a precipitate having an inositol/phosphorus ratio approximating that of inositol triphosphoric acid. In a subsequent paper Bower (11) found that yeast nucleic acid and phytic acid can be quantitatively

separated by precipitating the latter as the calcium salt under alkaline conditions. He applied this technique to sodium hydroxide extracts of soils and separated the soil organic phosphorus into two fractions. Both fractions were then compared to authentic phytin and yeast nucleic acid by determining their stability toward hypobromite oxidation and hydrolysis by specific enzymes. The agreement between the soil fractions and their authentic counterparts was very good.

The only data on the amount of inositol-bound phosphorus in the soil are those of Bower (10), who found that in three soils it comprised about 40 to 50 per cent of the total organic phosphorus, with phytin phosphorus making up about 70 to 75 per cent of the inositol-bound phosphorus. The relatively small amount of phosphorus in the form of the lower esters indicates that these are less stable in the soil than is the phytic acid itself.

The possible sources of phytin in the soil are plant residues, animal excrement, and microbial synthesis. Phytin appears to be present in all seeds in considerable quantity. Andrews and Bailey (2) found that in wheat bran phytin phosphorus made up 86 per cent of the total phosphorus, while in the germ it was only 48 per cent. However, DeTurk et al. (16) found that in the germination of corn the phytin was extensively hydrolyzed, although they did not con-

tinue their analyses long enough to see if hydrolysis was complete by the time of general decomposition of the seed. Thus it may be possible that despite phytase-induced hydrolysis the phytin in the outer seed tissues may be partially fixed by the soil. Furthermore, non-germinating seeds which rot in the soil may add their quota. Information on the presence of phytin in the vegetative parts of plants is scanty, although it appears to be agreed that if phytin is there it is found only in small amounts. DeTurk, Holbert and Howk (16) could not detect it in any vegetative part of the corn plant; Hart and Tottingham (28) did not find it in rutabagas or alfalfa hay or oat straw. Claims that it is present in the vegetative parts of plants are those of Tsuda (66) in clover hay, Knowles and Watkin (38) in wheat straw, Sundararajan (65) and Bagaoisan (4) in various leafy vegetables, and Binkley et al. (8) in cane juice. Some of the results on vegetables were not confirmed by Arbenz (3). The above determinations were all made by some modification of Heubner and Stadler's (31) iron precipitation method, and while it appears that this method is rather specific for phytin, it is not conclusively so. For instance, Leva and Rapoport (40) found that in determining phytin phosphorus in blood, the iron precipitates formed were always contaminated by other forms of organic phosphorus. Peperzak (53) made similar observations in work with faeces.

Thus, if there is phytin in the vegetative parts of the plant, it is probably in small amount. When one combines this low phytin content with Giri's (26) demonstration of the presence of phytase in the leafy parts of many plants, it seems reasonable to conclude that such phytin is not of great importance as a source of soil phytin. There is no direct evidence to support this conclusion, however.

Generalizations about animal excrement as a source of soil phytin are difficult even though phytin has repeatedly been shown to be present in faeces. The reason for this difficulty is the variability of the factors which can determine whether ingested phytin will be absorbed in the alimentary tract or not. Most of the information available has come from investigations into the rachitogenic nature of grain diets. Harrison and Mellanby (27) believe that this characteristic of such diets is due to their high phytic acid content. They found that neutral sodium phytate, phytic acid, and oatmeal produced rickets in puppies. Commercial phytin did not produce rickets and the addition of calcium to the sodium phytate or phytic acid diets reduced the severity of the rickets. Hence, they believe the problem is largely one of the balance between calcium and phytic acid. Calcium phytate is precipitated and excreted and the rickets are a result of the diminution in absorbable calcium (if phytic acid is present in sufficient amount) or absorbable

phosphorus (if phosphorus is mainly phytic and plenty of calcium is present). That oatmeal is strongly rachitogenic does not appear plausible at first glance, on the basis of this theory, since it is generally accepted that phytin is the saturated calcium-magnesium salt of phytic acid. Being base saturated, it should have no effect and should be excreted. They analyzed oatmeal for calcium and phytic phosphorus and found that less than half the calcium necessary to saturate the phytic acid was present. Hence, other cations, such as magnesium, sodium and potassium could be present which could exchange for calcium, thus limiting the amount of absorbable calcium. Support for this theory is provided by the work of Lowe and Steenbock (42), who found that autolyzed germinated corn was less rachitogenic than germinated corn. Further data on the phytic acid/calcium balance are given by Lowe and Steenbock (43), Cruickshank et al. (15), and Common (13), who found that in man, the rat, and poultry, while natural phytin was absorbed or hydrolyzed to varying degrees, the addition of calcium greatly increased the phytin in the faeces.

A further complication is the presence or absence of phytase in the alimentary tract or in ingested food. Where the phytin is partially soluble the presence of the enzyme will hasten solution by hydrolysis. Rather (58) fed pigs various grains and, using a method involving the precipitation of the strychnine salt, could detect no phytin in the faeces

except possibly where oats were fed. Schulerud (62) claims that oatmeal contains no phytase. This lack of enzyme might have been the cause of the recovery of phytin where oats were fed. Again Singsen and Mitchell (63) fed chicks soybean meal together with fresh alfalfa meal (presumably phytase active) or commercial alfalfa meal (phytase presumably killed by the heat treatment) and found that the utilization of the phytin was greater in the former case.

Hence it appears that the excretion of phytin may largely depend on the nature of the diet, and especially on the phytic acid/calcium ratio. Where both phytic acid and calcium are high, then phytin will probably be excreted in quantity. That such is the case is supported by the analysis of faeces of various animals. That from hens is always high as would be expected from the diet - grains high in phytin and calcium-containing material for the formation of shell. Peperzak (53), using an iron precipitation method of analysis, found that in chick and hen manure from various sources about 10 per cent of the total phosphorus was phytic. Kaila (36), who considered the organic phosphorus soluble in dilute acid as being predominantly phytic found 39 per cent of the phosphorus in fresh hen manure in this fraction. Both of these workers analyzed the dung from other domestic animals and found much less phytic phosphorus than in the case of hens. It was rarely above 10 per cent and commonly was around 0-4 per cent. Kaila (36) gives larger values

but his figures cannot be considered reliable for phytin due to the fractionation method used. Similar results are given by Ghani (25).

A few claims have been made that micro-organisms can synthesize phytin but none appears to be well authenticated. Kaila (36) found that 39 per cent of the phosphorus in Actinomyces violaceus ruber was dilute acid soluble organic phosphorus. He called this fraction phytin phosphorus but the criterion used is not specific. Macheboeuf et al. (45) claimed that phytin was present in the haptene fraction of the tubercle bacillus. They isolated a barium salt containing inositol and phosphorus. Common (13) found that 3.8 per cent of the phosphorus in dried yeast was phytic phosphorus. He used an iron precipitation as his method of isolation.

From the information available it seems probable that the main source of soil phytin is of plant origin, either from plant residues directly or via animals. However, synthesis by micro-organisms cannot be ruled out, especially since it appears that what work has been done on the fractionation of microbial phosphorus has seldom considered phytin phosphorus as a separate fraction. The rate of supply to the soil is undoubtedly small and the large amounts found in the soil are probably due to the great stability of these compounds in the soil.

The only direct studies of the fixation of inositol-

bound phosphorus are those of Bower (11), who used a modification of Heck's (29) method. Known equivalent amounts of phytin, "phytin derivatives" (i.e. lower phosphoric esters obtained by the partial hydrolysis of phytin) and monocalcium phosphate phosphorus, were added to soils having a pH range of 4.8 to 7.4. The recovery of the added phosphorus in an acid extractant of pH 3 was then determined. The fixation of all the forms of phosphorus increased progressively with the decrease of pH, the ranges of fixation being 7 to 32 per cent for monocalcium phosphate, 85 to 97 per cent for phytin and 57 to 86 per cent for phytin derivatives, with average figures of 15.6, 92.4, and 74.9 per cent, respectively. In agreement with Wrenshall and Dyer (71), Bower (11) considered that in the soil, phytin behaves very much as does inorganic phosphorus as far as fixation is concerned, although it is less soluble than inorganic phosphorus. At high pH values the phytic acid is present largely as the calcium salt, with a low but appreciable solubility; at low pH values the exceedingly insoluble iron and aluminum salts are formed. If this is so, then mineralization and availability of phytic phosphorus should decrease as the pH is lowered. This general tendency has been well authenticated. Bower (11) grew oats in the same soils used in the fixation experiment reported above and determined the response to and

recovery of phosphorus added to the soils in the form of phytin, phytin derivatives and monocalcium phosphate. Only soils with a pH above 6 showed a significant response to and recovery of phytin phosphorus, whereas all gave a response to monocalcium phosphate. The recovery of phytin phosphorus and its fixation were inversely related, the coefficient of correlation being -0.92. Where phytin derivatives were added the recovery was essentially nil in the case of two soils of pH 4.8 and 5.0, but with Webster soil of pH 7.4 it was almost as great as for monocalcium phosphate and about 3.5 times that for phytin.

Bower's findings generally corroborated those of previous workers who had investigated the mineralization of and plant response to phytin phosphorus. Rogers, Pearson and Pierre (59) found that corn and tomato plants grown in solutions could absorb phytin as such, the phosphorus intake being comparable to that from potassium dihydrogen phosphate. They found also that the roots of corn and tomato did not excrete any phytase. Whiting and Heck (67) grew oats in sand culture and found that the response to phytin was superior to the response to rock phosphate. Similar results were obtained by Heck and Whiting (30) with a following crop of red clover. Analyses made at the end of their experiment showed that all the phytin phosphorus had been mineralized. Neubauer (50) confirmed these results in the case of rye,

but when she added soil to her sand cultures, the response was greatly reduced or eliminated. Bertramson and Stephenson (7) found that in soils phytin was less available than other forms of organic phosphorus, all of which were less available than treble superphosphate. In an acid clay loam phytin was essentially unavailable. Similar results were obtained by Conrad (14).

Evidence that the decrease in phytin availability with decreasing soil pH may be largely due to reduced mineralization is given by the work of Pearson, Norman and Ho (52), who added phytin and various plant materials containing organic phosphorus to a soil of pH 5.83. A similar series had lime added such that the pH was 7.2. Both series were then incubated and the increase in inorganic phosphorus was measured. The addition of lime increased the mineralization of the organic phosphorus in all cases, but the effect on the added phytin phosphorus was striking as compared to the other forms. The increased mineralization due to lime was 3.5 per cent of the added phosphorus in the case of phytin, whereas with the other forms the effect of lime averaged 0.9 per cent.

It can be seen from these investigations that whereas in solution and sand cultures phytin phosphorus is readily available to plants, in soils the availability is greatly reduced, especially at low pH values. It is probable

that in soils this low availability at low p^H values is due to the increased fixation of the phytin which limits both the uptake of phytin as such by plants and the mineralization of the phytin phosphorus by micro-organisms.

THE SOLUBILITY AND PHYTASE CATALYZED HYDROLYSIS OF THE IRON, ALUMINUM, CALCIUM, AND MAGNESIUM SALTS OF PHYTIC ACID AND OF PHYTIC ACID DERIVATIVES.

The rate of hydrolysis of phytic acid depends on the level of enzyme activity and also on the concentration of the acid itself. In the soil this concentration will depend on the solubility of the various phytates which may exist there. The phytates present in the soil are probably mainly those of iron, aluminum, calcium and magnesium, their relative proportions depending partially on the soil pH value.

No systematic study has hitherto been made of the relative solubilities of these salts nor of their changes in solubility with change of pH. Yet the variation in response of plants to phytate phosphorus added to soils has been attributed solely to the formation of salts having different solubilities. As a basis for and as a check on past inferences a study of the solubilities of these salts was required. Furthermore, such information would be essential for evaluating the effectiveness of soil phytase in catalyzing the hydrolysis of phytin in soils. Accordingly, there was undertaken a series of experiments to

determine the solubilities of the phytates of the above metals together with experiments designed to show the relationship between the solubility of these phytates and their rate of hydrolysis by bran phytase. Since Bower (10) had shown that lower phosphoric acid esters of inositol existed in the soil, parallel runs were made using a mixture of these lower esters.

Solubility Studies

Materials and methods.

Preparation of sodium phytate. Technical calcium phytate, from the Corn Products Refining Co., New York, was dissolved in 2 per cent hydrochloric acid.* Insoluble material was filtered off and the filtrate was decolorized with charcoal. Excess solid calcium chloride was then added and when this had dissolved the calcium salt was precipitated by adding sodium hydroxide until the mixture was alkaline to litmus. The precipitate was filtered off on a Büchner funnel, washed with water and then dissolved in 8 per cent acetic acid and heated to boiling. The granular white precipitate which appeared was quickly filtered off

*All percentage concentrations of liquid reagents indicated herein are by volume of the concentrated reagent.

and washed with boiling water. Boutwell (9) has found that most of the inorganic phosphorus is removed by this treatment. This calcium salt was air dried and kept as a stock supply until required.

A pure solution of the sodium salt was prepared as follows. About 50 grams of the stock calcium phytate was dissolved in 2 per cent hydrochloric acid. Ferric phytate was then precipitated by adding a concentrated ferric chloride solution until the mixture showed a pale yellow tinge. This precipitate was filtered off and thoroughly washed on the filter with about 4 liters of 2 per cent hydrochloric acid. The washed salt was then suspended in water and sufficient sodium hydroxide was added to give a well flocculated precipitate of ferric hydroxide. The ferric hydroxide was filtered off and, after neutralizing the excess sodium hydroxide of the filtrate with hydrochloric acid, cupric acetate was added until the precipitation of the cupric phytate was complete. The cupric phytate was filtered off, washed with hot water, suspended in water and then decomposed with hydrogen sulfide. After filtering off the copper sulfide, the excess hydrogen sulfide was removed by aspiration over a period of about 12 hours. The resulting solution of phytic acid was titrated to pH 8 with sodium hydroxide and, after suitable dilution,

was used in the solubility studies.

The final pH of 8 was chosen to ensure stability of the solution without too great an excess of sodium. The yield was only about 50 per cent, but since large amounts of cheap technical calcium phytate were available no investigations concerning the improvement of the yield were undertaken.

In preliminary trials of various methods incorporating Boutwell's (9) boiling acetic acid technique, the purity of the final product was tested by determining the inositol/phosphorus ratio. The sample was sealed in a test tube with 2 to 3 N hydrochloric acid and heated for 14 hours at 140-150° C. The products were then analyzed for phosphorus and inositol using the method of Young (74). The theoretical ratio for inositol hexaphosphoric acid is 0.968. The ratio found was always higher, averaging 0.994 and indicating the presence of partially hydrolyzed derivatives of the hexaphosphoric acid. However, when an iron precipitation was included, followed by very thorough acid washing of the precipitate, the ratio was reduced and ranged from 0.966 to 0.974 in different preparations. The acid washing is essential. It appears to remove the iron salts of the lower phosphoric esters.

Preparation of sodium phytate derivatives. A solution of sodium phytate prepared by the above method was adjusted to pH 5.5 and incubated at room temperature with a phytase-active water extract of wheat bran. When analysis showed that about half the phosphorus was in inorganic form, the mixture was brought to the boiling point to inactivate any remaining enzyme and to coagulate the proteins from the bran extract. It was then filtered through charcoal and excess calcium chloride was added to the filtrate. The calcium salts were precipitated by adding sodium hydroxide until the mixture was alkaline to litmus. The precipitate was filtered off, washed with dilute sodium hydroxide and then dissolved in dilute nitric acid and further decolorized with charcoal. Most of the inorganic phosphorus was removed by precipitating it with ammonium molybdate according to the method of Willard and Diehl (69). This precipitate was filtered off. The final portion was extracted by shaking with isobutyl alcohol with excess molybdate present according to the method of Pons and Guthrie (56) for the determination of inorganic phosphorus. Only the inorganic phosphorus in the form of ammonium molybdiphosphate is extracted by the isobutyl alcohol. The calcium salts were then reprecipitated with sodium hydroxide, well washed with approximately 0.1 N sodium hydroxide and then dissolved in 10 per cent acetic acid. The lead salt was precipitated with excess lead

acetate, filtered off, washed with water and then suspended in water and decomposed with hydrogen sulfide. After filtering off the lead sulfide the excess hydrogen sulfide was removed by aspiration. The resultant acid solution was titrated to pH 8 with sodium hydroxide and used in the solubility studies. It had about 0.8 per cent of the total phosphorus in inorganic form and an inositol/phosphorus ratio of 2.14, which corresponds to 2.71 atoms of phosphorus per molecule.

The precipitation of the inorganic phosphorus required a large amount of ammonium molybdate. Some decomposition was probably caused by the strongly acid conditions. At the time this work was done the work of Plimmer and Page (55) was not appreciated. Their method would probably be an improvement. The lead salt was used in preference to the copper salt since Anderson (1) has shown that the lead salts of the lower esters are insoluble. It was not known whether the copper salts are insoluble to the same extent.

Solutions used.

Sodium phytate. A solution containing 3000 p.p.m. phytic phosphorus and 8 p.p.m. inorganic phosphorus.

Sodium phytate derivatives. A solution containing 900 p.p.m. inositol-bound phosphorus and 6.5 p.p.m. inorganic phosphorus.

Ferric chloride. Solutions containing 3.859 and 1.084 mg. iron/ml. were used for the phytate and

derivative studies, respectively. These were made by dissolving the reagent chemical in water and stabilizing the solution with a little hydrochloric acid. They were standardized by permanganate titration after the chloride had been removed with sulfuric acid.

Aluminum chloride. Solutions containing 1.806 and 0.5232 mg. aluminum/ml. were used for the phytate and derivative studies, respectively. The solutions were made by dissolving the reagent chemical in water. They were standardized gravimetrically using 8-hydroxyquinoline.

Calcium chloride. Solutions containing 3.83 and 1.164 mg. calcium/ml. were used for the phytate and derivative studies, respectively. A solution was made by dissolving reagent calcium carbonate in hydrochloric acid, filtering off a slight excess of carbonate and boiling out the carbon dioxide. This solution was standardized by oxalate precipitation followed by the weighing of the oxide and then was suitably diluted to give the above solutions.

Magnesium chloride. Solutions containing 3.02 and 0.7066 mg. magnesium/ml. were used for phytate and derivative studies, respectively. The solutions were made by dissolving the reagent chemical in water and were standardized gravimetrically using 8-hydroxyquinoline.

Sodium hydroxide and hydrochloric acid. Both approximately normal solutions.

Method. Five ml. of the sodium phytate or sodium phytate derivatives solutions were pipetted into 500-ml. Erlenmeyer flasks and followed by the necessary amounts of sodium hydroxide or hydrochloric acid, as found previously, to give the required pH. Then the necessary amount of metallic chloride solution was added followed by water to bring the volume to 300 ml. and 200 ml. for the phytate

and phytate derivatives series, respectively. A few drops of toluene were added to inhibit bacterial action and the flasks were then stoppered. The contents were well mixed and the flasks were then allowed to stand for 3 to 11 days with occasional shaking. The temperature was 26±2° C. The flasks were then opened and the pH was found on one portion using a glass electrode. Another portion was filtered and the phosphorus content of the filtrate was determined by ashing with magnesium nitrate and measuring the resultant inorganic phosphorus by the colorimetric method of Dickman and Bray (17).

Although carbon dioxide was not rigorously excluded, all solutions were made up in boiled water and during filtration the samples were protected with Ascarite tubes. The concentrations of phosphorus were 50 p.p.m. (.00028 M) and 22.5 p.p.m. (.00027 M) for the phytate and derivatives, respectively. The amounts of the metallic cations used were 1, 3.75 and 6.25 times the equivalence of the phosphate groups present. The 3.75 times equivalence was based on Harley's (19) findings that until this excess was present, the insoluble salt formed was largely the triferric salt but that with a larger excess it was the tetraferric salt. The larger excess was included so as to approach more nearly the proportion likely to be found in the soil.

Equilibrium had apparently been reached since reanal-

ysis at a later date showed only very slight changes in soluble phosphorus. In a few cases, especially with the calcium salt, a change was found, but a parallel change in pH was also found such that when solubility was plotted against pH, the new points fell on the same curve as found by previous analysis. Hoff-Jørgensen (32) found that equilibrium was reached very quickly in the case of calcium phytate.

Gaarder (24), in similar work on inorganic phosphates, used an ultra filter to separate his precipitates. Such equipment was not available and various methods of filtering were tried. Macerated filter paper, fine asbestos, an atmometer cup and Selas fine sintered crucibles were all found unsatisfactory. Finally a layer of acid-washed, ball-milled, quartz sand supported on sintered glass crucibles was used. This procedure was satisfactory and gave clear filtrates except in the case of the ferric phytate precipitates at pH values greater than 8 and ferric phytate derivatives at pH values greater than 7. If these pH values were exceeded the ferric hydroxide was dispersed to such an extent that some passed into the filtrate. The ball-milled quartz was considered to be inert for the purpose for which it was used since it allowed soluble sodium phytate to pass through quantitatively and with no change in pH. This point was verified by discarding the quartz for the alternate members

of one of the magnesium phytate series. Irrespective of whether the quartz was present or not, all the results fitted the same pH-solubility curve.

Results and discussion.

The solubility data are given in Tables 1 to 12 and Figures 1 to 4. Inspection of these results shows three main features. Firstly, there is the contrast between the solubilities of the iron and aluminum phytates and phytate derivatives on the one hand and the calcium and magnesium salts on the other. The former are characterized by their insolubilities at low pH values and the latter by their insolubilities at pH values greater than 5. Secondly, there is the general greater solubility of the phytate derivatives as compared to the corresponding phytates, especially in the case of the calcium and magnesium salts. Thirdly, there is the effect of the increase in the amount of the metallic cation present. In the case of the calcium and magnesium salts, the increase in cation concentration causes a decrease in solubility as would be expected from the common-ion principle. With iron and aluminum, increase in the cation concentration at low pH values causes either little change in solubility or an increased solubility.

The present work confirms the conclusions of previous

Table 1. The solubility of ferric phytate and ferric phytate derivatives at different pH values with the iron to phytate phosphorus equivalent ratio of 1.

Final pH	Soluble phosphorus	
	Milligrams	Per cent of total
-Ferric phytate*-		
0.85	0.27	1.8
1.20	0.06	0.4
1.50	0.03	0.2
1.95	0.03	0.2
2.50	0.03	0.2
3.00	0.03	0.2
3.20	0.06	0.4
3.70	0.12	0.8
4.50	1.14	7.6
5.40	2.13	14.2
5.60	2.34	15.6
6.70	3.60	24.0
9.70	6.84	45.6
-Ferric phytate derivatives-**		
1.00	2.23	49.6
1.46	0.67	14.8
1.95	0.40	8.9
2.55	0.34	7.5
2.72	0.34	7.5
2.99	0.42	9.3
3.00	0.46	10.2
3.13	0.46	10.2
3.30	0.52	11.5
3.60	0.64	14.2
3.85	0.72	16.0
4.15	1.01	22.4
4.75	2.22	49.3
4.80	2.28	50.6
5.25	2.44	54.2
5.90	2.64	58.7
6.78	3.10	68.9
6.84	3.22	71.5
7.32	3.32	73.8

*Phytate P present, 15 mg.; iron present, 18.02 mg.
**Phytate derivative P present, 4.5 mg.; iron present, 5.42 mg.

Table 2. The solubility of ferric phytate at different pH values with the iron to phytate phosphorus equivalent ratio of 3.75.*

Final pH	Soluble phosphorus	
	Milligrams	Per cent of total
1.20	11.46	76.4
1.60	4.44	29.6
1.80	2.04	13.6
2.30	0.18	1.2
2.55	0.03	0.2
2.90	0.03	0.2
3.60	0.03	0.2
4.60	0.06	0.4
6.90	0.00	0.0
7.20	0.09	0.6
7.95	0.48	3.2
8.35	1.62	10.8
9.40	6.06	40.8

*Phytate P present, 15 mg.; iron present, 67.61 mg.

Table 3. The solubility of ferric phytate and ferric phytate derivatives at different pH values with the iron to phytate phosphorus equivalent ratio of 6.25.

Final pH	Soluble phosphorus	
	Milligrams	Per cent of total
	-Ferric phytate*-	
1.20	13.10	87.3
1.30	4.80	32.0
1.50	1.44	9.6
2.20	0.09	0.6
2.30	0.03	0.2
3.10	0.03	0.2
3.80	0.03	0.2
4.70	0.03	0.2
6.10	0.03	0.2
6.80	0.09	0.6
7.50	0.03	0.2
8.50	0.06	0.4
9.75	1.65	11.0
9.90	1.38	9.2
	-Ferric phytate derivatives**-	
2.26	3.00	66.7
2.40	2.34	52.0
2.50	2.03	45.1
2.79	0.09	2.0
2.95	0.09	2.0
3.25	0.02	0.4
3.50	0.02	0.4
3.80	0.02	0.4
4.18	0.01	0.2
6.22	0.04	0.8
6.90	0.36	8.0
7.18	1.06	23.5

*Phytate P present, 15 mg.; iron present, 112.68 mg.
**Phytate derivative P present, 4.5 mg.; iron present, 33.88 mg.

Table 4. The solubility of aluminum phytate and aluminum phytate derivatives at different pH values with the aluminum to phytate phosphorus equivalent ratio of 1.

Final pH	Soluble phosphorus	
	Milligrams	Per cent of total
	-Aluminum phytate*-	
1.55	15.06	100.0
1.90	4.02	26.8
2.15	0.72	4.8
2.65	0.09	0.6
3.10	0.05	0.3
3.45	0.03	0.2
3.95	0.05	0.3
4.60	0.26	1.6
5.25	0.60	4.0
6.40	2.10	14.0
7.30	3.72	24.8
8.30	5.55	37.0
8.90	7.32	48.8
9.45	11.25	75.0
	-Aluminum phytate derivatives**-	
2.62	3.4	77.0
2.81	1.92	42.5
3.04	1.52	33.8
3.17	1.36	30.2
3.40	1.12	25.0
3.60	1.04	23.1
3.90	1.02	22.7
4.50	0.96	21.2
4.55	0.86	19.1
4.62	0.96	21.2
4.97	1.36	30.2
5.58	2.70	60.0
6.15	3.28	73.0
6.25	3.40	77.0
7.05	3.60	80.0

*Phytate P present 15 mg.; aluminum present, 8.70 mg.
**Phytate derivative P present, 4.5 mg.; aluminum present, 2.62 mg.

Table 5. The solubility of aluminum phytate at different pH values with the aluminum to phytate phosphorus equivalent ratio of 3.75.*

Final pH	Soluble phosphorus	
	Milligrams	Per cent of total
1.55	9.51	63.4
1.90	1.65	11.0
2.10	0.36	2.4
3.00	0.06	0.4
3.20	0.00	0.0
3.95	0.00	0.0
4.30	0.03	0.2
4.50	0.03	0.2
7.80	0.05	0.3
9.00	0.14	0.9
9.65	2.97	19.8
9.90	9.2	61.3

*Phytate P present, 15 mg.; aluminum present, 32.65 mg.

Table 6. The solubility of aluminum phytate and aluminum phytate derivatives at different pH values with the aluminum to phytate phosphorus equivalent ratio of 6.25.

Final pH	Soluble phosphorus	
	Milligrams	Per cent of total
	-Aluminum phytate*-	
1.65	12.72	84.8
1.80	5.25	35.0
2.00	0.60	4.0
2.20	0.27	1.8
2.90	0.12	0.8
3.40	0.06	0.4
3.90	0.00	0.0
4.10	0.00	0.0
5.50	0.00	0.0
7.30	0.05	0.3
9.00	0.03	0.2
9.50	0.15	1.0
9.85	0.68	4.5
9.95	3.90	26.0
	-Aluminum phytate derivatives**-	
3.10	4.07	90.5
3.60	3.12	71.0
3.85	1.46	32.5
4.00	0.56	12.4
4.14	0.20	4.4
4.23	0.04	0.9
4.43	0.00	0.0
4.75	0.01	0.2
5.46	0.01	0.2
7.43	0.00	0.0
8.90	0.23	5.1
9.10	0.56	12.4

* Phytate P present, 15 mg.; aluminum present, 54.31 mg.
**Phytate derivative P present, 4.5 mg.; aluminum present, 15.35 mg.

Table 7. The solubility of calcium phytate and calcium phytate derivatives at different pH values with the calcium to phytate phosphorus equivalent ratio of 1.

Final pH	Soluble phosphorus	
	Milligrams	Per cent of total
	-Calcium phytate*-	
5.40	15.06	100.0
5.52	13.14	87.6
5.55	12.15	81.0
5.64	9.03	60.0
5.98	3.60	24.0
6.62	0.69	4.6
6.80	0.51	3.4
8.20	0.09	0.6
8.50	0.06	0.4
9.20	0.06	0.4
10.00	0.27	1.8
	-Calcium phytate derivatives**-	
7.15	4.24	94.3
7.66	3.34	74.2
8.55	2.86	63.5
10.15	2.84	63.1
10.50	2.76	61.4

*Phytate P present, 15 mg.; calcium present, 19.41 mg.
**Phytate derivative P present, 4.5 mg.; calcium present, 5.82 mg.

Table 8. The solubility of calcium phytate at different pH values with the calcium to phytate phosphorus equivalent ratio of 3.75.*

Final pH	Soluble phosphorus	
	Milligrams	Per cent of total
4.88	14.04	93.6
4.90	11.97	79.8
4.93	11.31	75.4
5.04	8.31	55.4
5.55	0.60	4.0
6.45	0.09	0.6
7.10	0.06	0.4
9.40	0.06	0.4
9.80	0.03	0.2
10.20	0.03	0.2
10.60	0.09	0.6

*Phytate P present, 15 mg.; calcium present, 42.73 mg.

Table 9. The solubility of calcium phytate and calcium phytate derivatives at different pH values with the calcium to phytate phosphorus equivalent ratio of 6.25.

Final pH	Soluble phosphorus	
	Milligrams	Per cent of total
	-Calcium phytate*-	
4.48	15.06	100.0
4.78	13.92	92.8
4.81	11.64	77.6
4.90	7.89	52.6
5.39	0.60	4.0
6.06	0.06	0.4
7.02	0.06	0.4
9.16	0.06	0.4
9.82	0.06	0.4
10.10	0.02	0.1
10.58	0.03	0.2
	-Calcium phytate derivatives**-	
6.25	3.38	75.0
7.70	1.88	41.7
9.95	1.60	35.6
10.28	1.62	36.0
10.50	1.54	34.2

*Phytate P present, 15 mg.; calcium present, 121.26 mg.
**Phytate derivative P present, 4.5 mg.; calcium present, 36.37 mg.

Table 10. The solubility of magnesium phytate and magnesium phytate derivatives at different pH values with the magnesium to phytate phosphorus equivalent ratio of 1.

Final pH	Soluble phosphorus	
	Milligrams	Per cent of total
	-Magnesium phytate*-	
7.05	14.28	95.2
7.23	10.74	71.6
7.35	7.47	49.8
7.76	4.35	29.0
8.30	1.56	10.4
9.35	0.96	6.4
9.93	0.87	5.8
10.30	0.84	5.6
	-Magnesium phytate derivatives**-	
8.82	4.48	99.6
10.2	4.26	94.7

*Phytate P present, 15 mg.; magnesium present, 11.78 mg.
**Phytate derivative P present, 4.5 mg.; magnesium present, 3.53 mg.

Table 11. The solubility of magnesium phytate at different pH values with the magnesium to phytate phosphorus equivalent ratio of 3.75.*

Final pH	Soluble phosphorus	
	Milligrams	Per cent of total
6.20	15.3	100.0
6.50	13.11	87.4
6.60	8.88	59.2
6.78	5.10	34.0
6.95	2.22	14.9
7.28	0.54	3.6
8.70	0.18	1.2
9.05	0.15	1.0
9.30	0.12	0.8
9.70	0.06	0.4
10.00	0.03	0.2

*Phytate P present, 15 mg.; magnesium present, 44.15 mg.

Table 12. The solubility of magnesium phytate and magnesium phytate derivatives at different pH values with the magnesium to phytate phosphorus equivalent ratio of 6.25.

Final pH	Soluble phosphorus	
	Milligrams	Per cent of total
	-Magnesium phytate*-	
6.15	14.94	99.6
6.41	13.35	89.0
6.70	4.95	33.0
6.82	2.79	18.6
6.90	1.59	10.6
7.25	0.87	5.8
7.70	0.57	3.8
9.27	0.12	0.8
9.75	0.06	0.4
10.00	0.03	0.2
	-Magnesium phytate derivatives**-	
8.90	4.28	95.0
9.18	4.12	91.5
9.35	4.10	91.0
9.62	3.44	76.5
9.90	3.20	71.0
10.09	2.88	64.1
10.15	2.64	58.6

* Phytate P present, 15 mg.; magnesium present, 73.71 mg.
**Phytate derivative P present, 4.5 mg.; magnesium present, 22.97 mg.

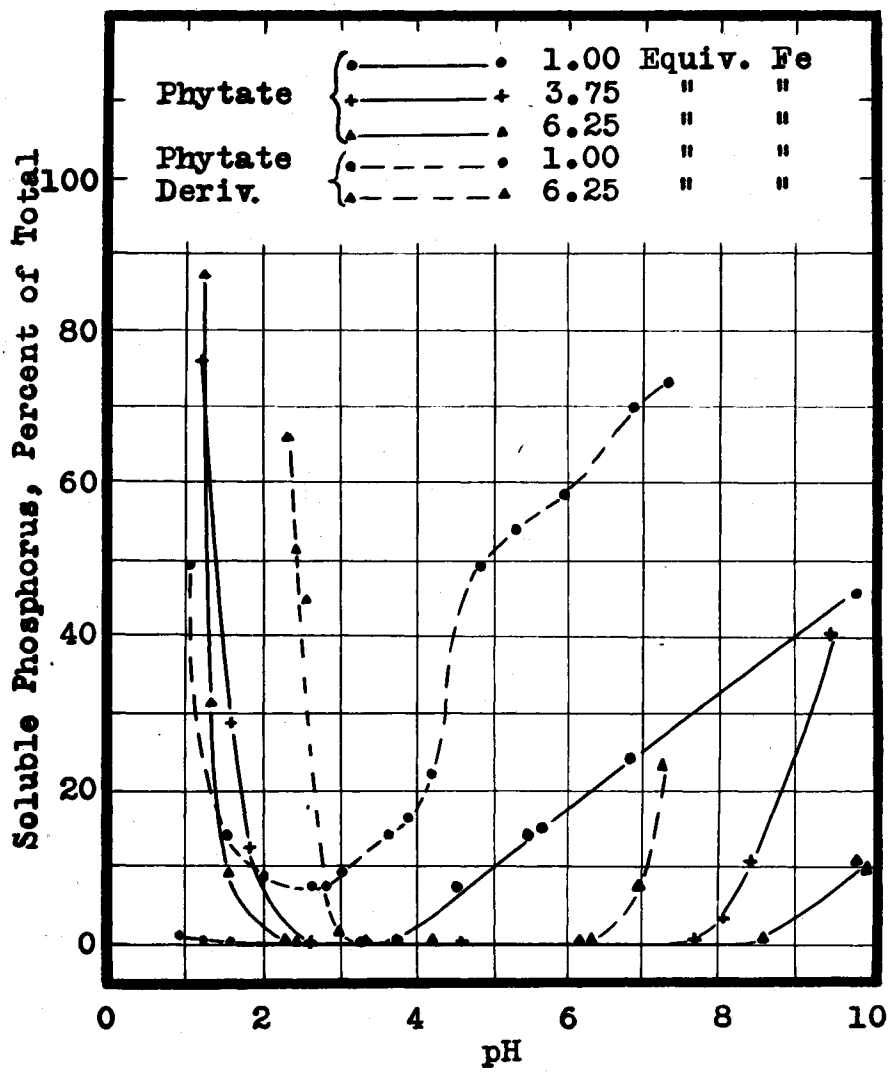

Figure 1. Solubility of ferric phytate and ferric phytate derivatives at different pH values with varying iron to phytate phosphorus equivalent ratios.

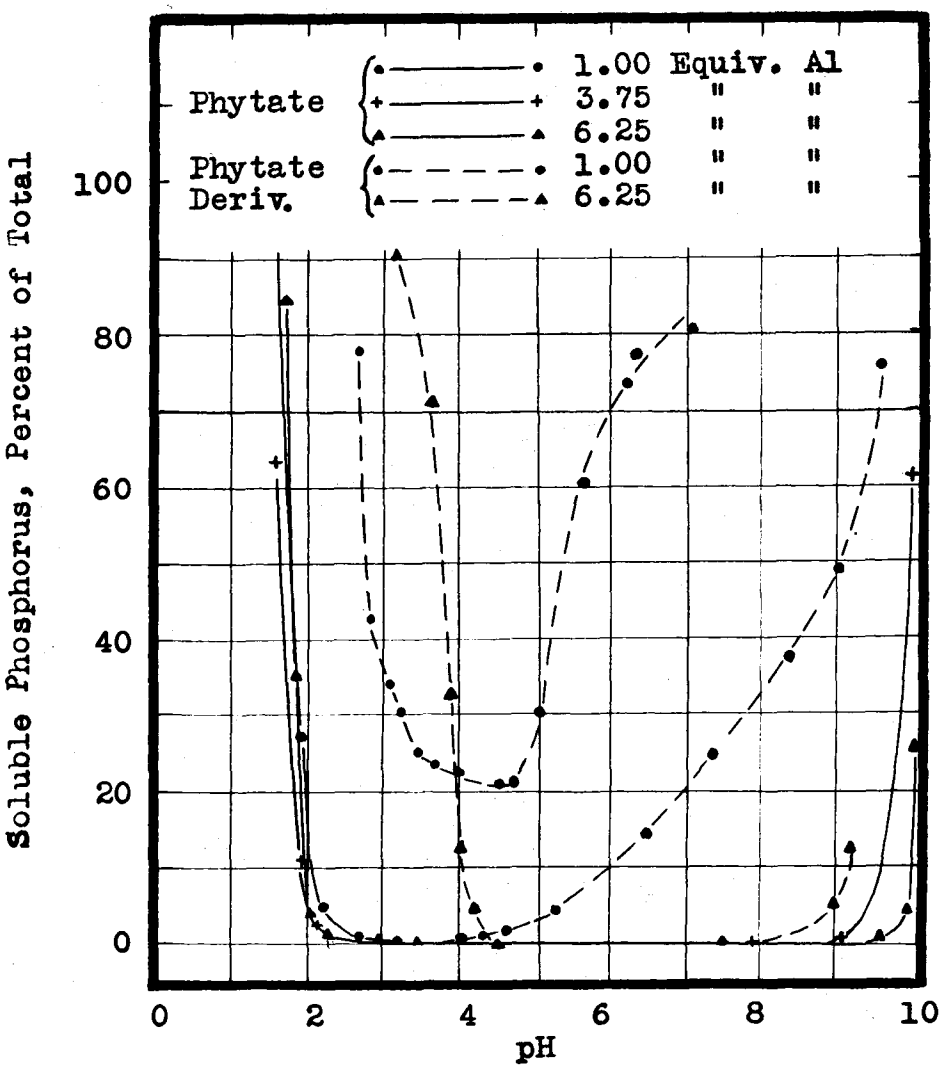

Figure 2. Solubility of aluminum phytate and aluminum phytate derivatives at different pH values with varying aluminum to phytate phosphorus equivalent ratios.

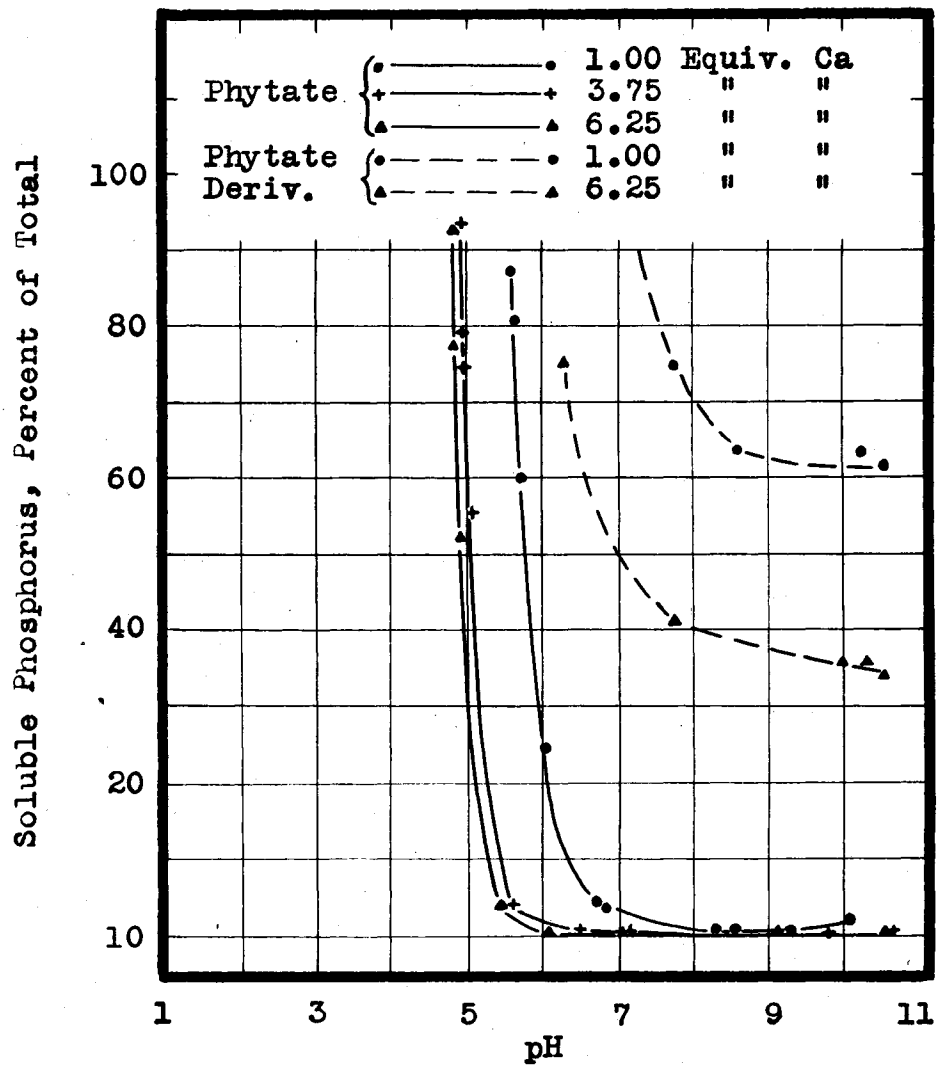

Figure 3. Solubility of calcium phytate and calcium phytate derivatives at different pH values with varying calcium to phytate phosphorus equivalent ratios.

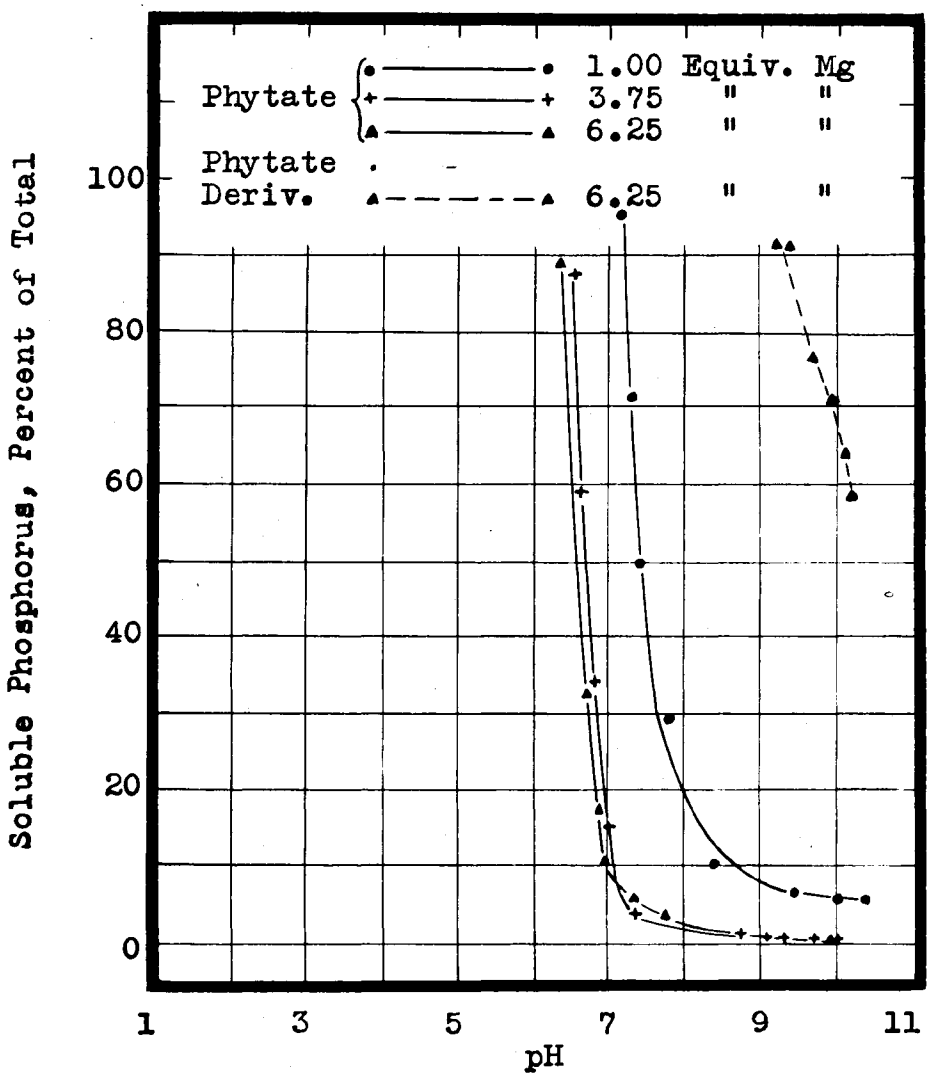

Figure 4. Solubility of magnesium phytate and magnesium phytate derivatives at different pH values with varying magnesium to phytate phosphorus equivalent ratio.

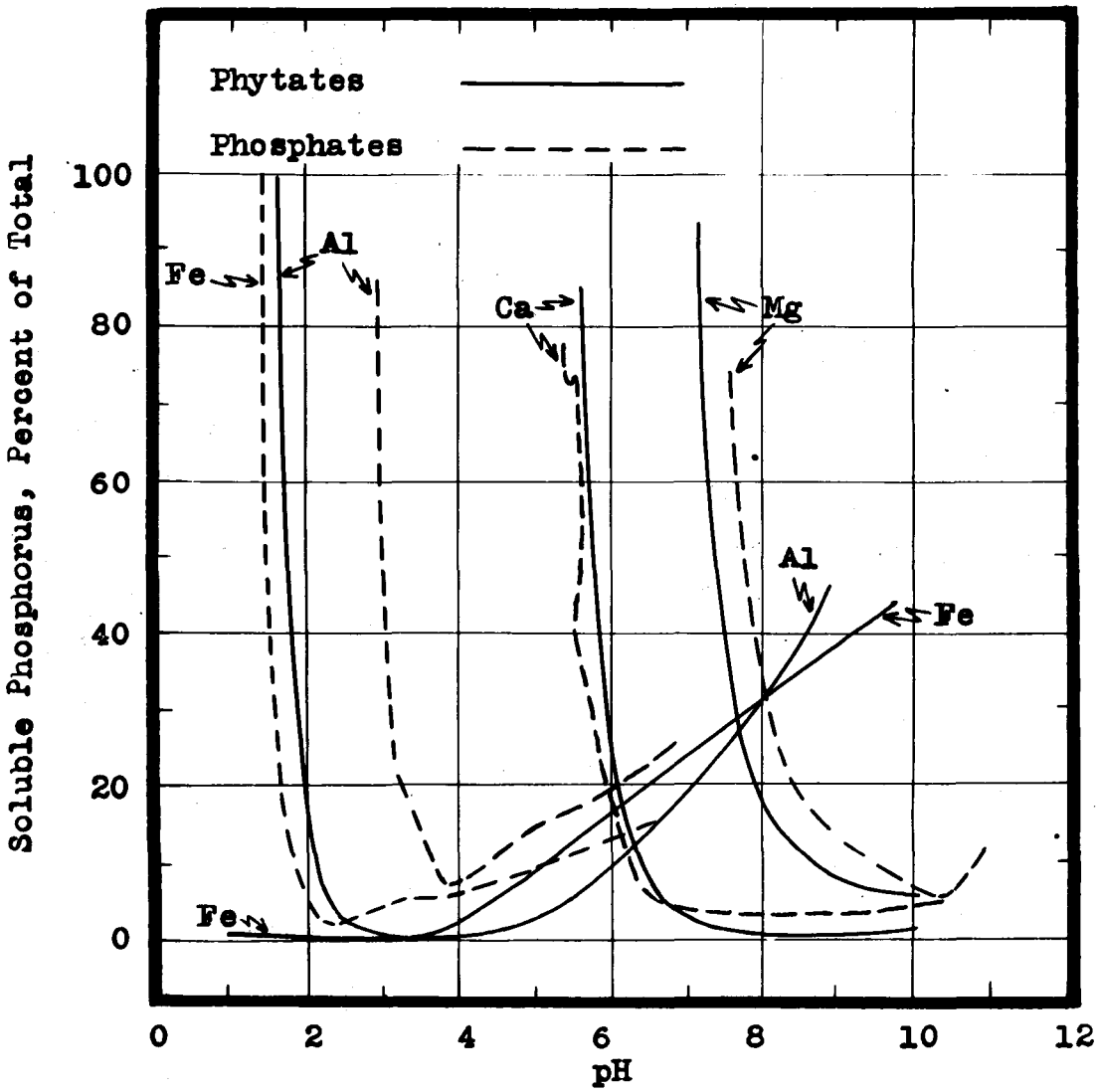

Figure 5. Solubility of iron, aluminum, calcium and magnesium phytates and phosphates at different pH values with cation to phosphorus equivalent ratio of 1.

workers that the ferric and aluminum phytates are very insoluble at low pH values. An anomoly is seen in the case of ferric phytate. At pH values less than 2 and with an excess of iron present the apparent solubility is much greater than when an equivalent amount of iron is present. This peculiarity may be explained by the formation, when excess iron is present, of relatively stable supersaturated solutions and/or stable complexes. In work subsequent to that reported in the tables it was found that at pH 1.7 and with excess iron present, a solution of sodium phytate could be boiled without any precipitation taking place. However if sodium sulfate was then added, precipitation was immediate and complete. This effect of the added salt suggests that the high solubility is due to the formation of stable supersaturated solutions. The high solubility is essentially spurious.

That the formation of soluble complexes may be a contributing cause of the high solubility is suggested by Hoff-Jørgensen's (32) work on the extent of complex formation in calcium phytate. He found that it was considerable and that as the relative amount of calcium was increased the proportion of the calcium in the complexed form increased as compared to that in true phytate form. It is possible that complex formation amongst phytates is common, especially when excess cations are present, and that under these con-

ditions this complexing may play an important part in causing the relatively high solubility of ferric phytate. It is not known if the high solubility of the aluminum phytate is similarly spurious. In this case increasing the cation concentration had little effect on the solubility.

The only minimum points of solubility for the ferric and aluminum phytates and phytate derivatives that can be estimated are for the systems where the cations and phytate phosphorus are present in equivalent quantities. With ferric phytate, this minimum lies between pH 2 and pH 3 and with aluminum phytate between pH 3 and pH 4. This shift in the point of minimum solubility also applies to the derivatives, that for the ferric salt being at pH 2.5 and that for the aluminum salt at pH 4.5. With excess cations present the ferric phytate is insoluble from approximately pH 2.5 to pH 8 and the aluminum salt is insoluble from approximately pH 3 to pH 9. Under similar conditions the ferric phytate derivatives are insoluble from pH 3.5 to pH 6.5 and the aluminum salts are insoluble from pH 4.5 to pH 8.

The irregularities noted above are absent from the calcium and magnesium systems. With an equivalent amount of calcium present the phytate shows a point of minimum solubility at pH 9, when it is essentially all precipitated. The slight increase in solubility at pH 10 may be due to

the formation of the more soluble mixed calcium-sodium salt. With excess calcium present full insolubility begins at approximately pH 6.5. The calcium phytate derivatives are always appreciably soluble. The magnesium systems are very similar to those for calcium but the solubility is always higher. Essentially full insolubility occurs only with the phytate at pH 10 when there is an excess of magnesium present.

The only work with which this can be compared is that on the solubility of calcium phytate carried out by Hoff-Jørgensen (32). From his data it appears that the calcium salt is much less soluble than would appear from the present work. He was able to obtain precipitates at pH values as low as 2.8, but above pH 5.5 the solubilities appear to be about the same. Although it is difficult to get an adequate picture from his paper it would appear that the slope of his solubility curve below pH 5 is much flatter than is shown in Figure 3. A possible explanation for this is that whereas the present work was carried out at a temperature of 26° C, Hoff-Jørgensen held his systems at 37° C. Increasing the temperature greatly decreases the solubility of calcium phytate. A further possibility is that the phytate concentrations used in the present investigation were too low. Calculations based on Hoff-Jørgensen's solubility products showed that at pH 5 its value was exceeded in the present

systems used. However the determinations from which he calculated his solubility products were made at 37° C and might be considerably larger at 26° C due to the large temperature effect.

It is of interest to compare the results of the present investigation with those of Gaarder (24) who, under very similar conditions, determined the solubilities of iron, aluminum, calcium and magnesium phosphates. In Figure 5 are given the solubility curves for iron, aluminum, calcium and magnesium phosphates and phytates when the cations are present in amounts equivalent to the phosphorus present. The curves for the phosphates are redrawn from Gaarder's publication. The main difference in the systems used was that Gaarder had an 0.0055 M concentration of phosphate whereas in the present studies the phytate concentration was 0.00028 M. These concentrations are 0.0165 N and 0.00336 N, respectively; hence, the difference in concentrations of the active phosphate groups is not extreme.

The general similarity between the phosphate and phytate solubilities is evident. The one dissimilarity is the insolubility of the ferric phytate below pH 2. The similarity between the calcium salts was unexpected since at pH values below 6.8 the phosphate is less soluble than the phytate and Hoff-Jørgensen (32) found that the reverse was the case. This behavior supports the belief that the concentration of

phytate used was too low and that at low pH values the solubility product was not exceeded due to this cause. This condition probably applies to the magnesium salt also.

Despite the low concentrations of phytate used it can be seen that with the iron, aluminum and calcium systems the minimum solubility of the phytates is significantly less than that of the phosphates, which are not fully insoluble at any pH. In the soil it is probably these small solubilities and the differences between them which are of importance in determining the extent of availability of these various forms of phosphorus to plants. As with the inorganic phosphorus, it appears that the pH range over which the solubility is greatest for phytate phosphorus is from pH 6.0 to pH 6.5. Thus in so far as the availability of phytin phosphorus to plants is determined by its solubility, it appears that agronomic practices designed to maintain the soil pH within this range would be most desirable.

Hydrolysis Studies

Materials and methods.

Preparation of phytase extract. Wheat bran and water (1:10) were shaken for 30 minutes and were then filtered through cloth and finally through paper. Two volumes of

95 per cent ethanol were then added and the resulting precipitate was filtered off, redissolved in water and, after the insoluble material had been filtered off, again precipitated with ethanol. After filtering, the precipitate was washed with ethanol and ether and dried on a porous plate. It was then ground to a fine, light-brown powder and stored in a closed bottle in the refrigerator.

The stability of this suspension was tested since it was planned to use the same preparation over a period of several hours. A sample was made up and kept in the refrigerator. After various periods of time up to 10 hours a constant amount of the suspension was added to identical samples of sodium phytate. These were then incubated at 47° C and, after increasing periods of time, samples were withdrawn and analyzed for inorganic phosphorus. It was found that in all cases the curves obtained by plotting the per cent hydrolysis against time of incubation, were essentially the same and showed no decrease in phytase activity with increase in the age of the phytase when added to the sodium phytate.

Solutions used. The following solutions were prepared and standardized as described in the previous section.

Sodium phytate. A solution having 250 p.p.m. phytic phosphorus.

Sodium phytate derivatives. A solution having 133.3 p.p.m. inositol-bound phosphorus with 3.2 atoms of phosphorus per molecule.

Ferric chloride. Solution (1), 0.751 mg. Fe/ml. Solution (2), 3.131 mg. Fe/ml.

Aluminum chloride. A solution having 1.134 mg. Al/ml.

Calcium chloride. A solution having 2.527 mg. Ca/ml.

Magnesium chloride. A solution having 1.534 mg. Mg/ml.

Hydrochloric acid and sodium hydroxide. Approximately 0.01 and 0.03 N solutions of both.

Phytase suspension. 0.5 grams of the powdered extract triturated with a little water and made up to 600 ml.

Method. Ten ml. of the sodium phytate or sodium phytate derivatives solutions followed by the amounts of distilled water necessary to give a final volume of 54 ml. were added to series of 2-ounce, screw-capped, medicine bottles. Then hydrochloric acid or sodium hydroxide was added in amounts necessary to give the desired pH values. Finally the bottles received the required amounts of the metallic chloride solutions. The bottles were then capped and placed in an oven at $45 \pm \frac{1}{2}^{\circ}$ C. When the bottles had reached oven temperature, 3 ml. of the well-shaken phytase suspension were added to successive bottles at 1 minute intervals. After 3 hours' incubation, the bottles were removed at 1 minute intervals in the order of the addition of the phytase

extract and aliquots were pipetted into test tubes containing 2 drops of concentrated hydrochloric acid. These were then placed in the refrigerator and later analyzed for inorganic phosphorus by the isobutyl alcohol extraction method of Pons and Guthrie (56). The pH was found using the remainder of the mixture.

The final concentrations of phosphorus were 46.3 p.p.m. and 24.7 p.p.m. of phytate and phytate derivative phosphorus, respectively. The iron was added in amounts 1.00 and 6.25 times the equivalent amount of phosphorus present. The other cations were added at the higher level only.

A trial run showed that the storage of the samples for analysis with two drops of hydrochloric acid, did not result in any hydrolysis during the period of storage. The method of Pons and Guthrie (56) was selected for analysis since no filtering is required and since the high amounts of ferric and aluminum ions present would have interfered had the method of Dickman and Bray (17) been used. The isobutyl alcohol extraction eliminates the interference of such substances. Owing to the large number of samples, only one analysis was made per sample.

Results and discussion.

The results are given in Tables 13 to 17 and in Figure 6. The data for aluminum are omitted from Figure 6 owing to the difficulty of representing them clearly.

Table 13. The hydrolysis of ferric phytate and ferric phytate derivatives of different pH values with the iron to phytate phosphorus equivalent ratio of 1.

Final pH	Total inorganic P measured, mg	Inorganic P added,* mg	Phosphorus hydrolyzed mg	Phosphorus hydrolyzed per cent
		-Ferric phytate**-		
4.1	0.184	0.167	0.017	0.7
4.4	0.184	0.167	0.017	0.7
5.0	0.178	0.167	0.011	0.4
5.7	0.178	0.167	0.011	0.4
6.6	0.178	0.167	0.011	0.4
7.1	0.206	0.167	0.039	1.5
		-Ferric phytate derivatives***-		
4.2	0.189	0.173	0.016	1.2
4.5	0.189	0.173	0.016	1.2
4.8	0.227	0.173	0.054	4.1
5.7	0.205	0.173	0.032	2.4
6.7	0.308	0.173	0.135	10.2
6.9	0.298	0.173	0.125	9.4

*Mainly added with enzyme
**Phytate P present, 2.5 mg.; iron present, 3.004 mg. (4 ml. of solution 1)
***Phytate derivative P present, 1.33 mg.; iron present, 1.61 mg. (2.13 ml. of solution 1)

Table 14. The hydrolysis of ferric phytate and ferric phytate derivatives at different pH values with the iron to phytate phosphorus equivalent ratio of 6.25.

Final pH	Total inorganic P measured, mg	Inorganic P added,* mg	Phosphorus hydrolyzed mg	Phosphorus hydrolyzed per cent
		-Ferric phytate**-		
3.6	0.189	0.167	0.022	0.9
4.1	0.157	0.167	-0.010	-0.4
4.5	0.200	0.167	0.033	1.3
5.5	0.178	0.167	0.011	0.4
6.6	0.211	0.167	0.044	1.8
7.2	0.184	0.167	0.017	0.7
		-Ferric phytate derivatives***-		
3.4	0.221	0.173	0.048	3.6
3.7	0.200	0.173	0.027	2.0
4.6	0.211	0.173	0.038	2.9
6.4	0.227	0.173	0.054	4.1
6.9	0.189	0.173	0.016	1.2
7.2	0.200	0.173	0.027	2.0

*Mainly added with enzyme
**Phytate P present, 2.5 mg.; iron present, 18.786 mg. (6 ml. of solution 2)
***Phytate derivative P present, 1.33 mg.; iron present, 10.029 mg. (3.2 ml. solution 2)

Table 15. The hydrolysis of aluminum phytate and aluminum phytate derivatives at different pH values with the aluminum to phytate phosphorus equivalent ratio of 6.25.

Final pH	Total inorganic P measured, mg	Inorganic P added,* mg	Phosphorus hydrolyzed mg	Phosphorus hydrolyzed per cent
		-Aluminum phytate**-		
3.6	0.173	0.167	0.006	0.2
4.0	0.173	0.167	0.006	0.2
5.0	0.157	0.167	-0.010	-0.4
6.3	0.227	0.167	0.060	2.4
7.4	0.200	0.167	0.033	1.3
7.8	0.184	0.167	0.017	0.7
		-Aluminum phytate derivatives***-		
4.0	0.167	0.173	-0.006	-0.4
4.5	0.167	0.173	-0.006	-0.4
4.7	0.167	0.173	-0.006	-0.4
6.9	0.162	0.173	-0.11	-0.8
7.2	0.173	0.173	0.00	0.0

*Mainly added with enzyme
**Phytate P present, 2.5 mg.; aluminum present, 9.072 mg. (8 ml. of solution)
***Phytate derivative P present, 1.33 mg.; aluminum present, 4.831 mg. (4.26 ml. of solution)

Table 16. The hydrolysis of calcium phytate and calcium phytate derivatives at different pH values with the calcium to phytate phosphorus equivalent ratio of 6.25.

Final pH	Total inorganic P measured, mg	Inorganic P added,* mg	Phosphorus hydrolyzed	
			mg	per cent
		-Calcium phytate**-		
3.8	0.427	0.167	0.260	10.4
5.2	0.961	0.167	0.794	31.7
5.6	0.621	0.167	0.454	18.2
5.8	0.454	0.167	0.277	11.2
6.2	0.400	0.167	0.233	9.3
6.6	0.329	0.167	0.162	6.5
		-Calcium phytate derivatives***-		
3.8	0.562	0.173	0.389	29.2
4.4	0.664	0.173	0.491	36.9
5.7	0.869	0.173	0.696	52.3
6.3	0.772	0.173	0.599	45.0
6.8	0.535	0.173	0.362	27.2
7.0	0.308	0.173	0.135	10.2

*Mainly added with enzyme
**Phytate P present, 2.5 mg.; calcium present, 20.216 mg. (8 ml. of solution)
***Phytate derivative P present, 1.33 mg.; calcium present, 10.765 mg. (4.26 ml. of solution)

Table 17. The hydrolysis of magnesium phytate and magnesium phytate derivatives at different pH values with the magnesium to phytate phosphorus equivalent ratio of 6.25.

Final pH	Total inorganic P measured, mg	Inorganic P added,* mg	Phosphorus hydrolyzed mg	Phosphorus hydrolyzed per cent
		-Magnesium phytate**-		
3.9	0.486	0.167	0.319	12.8
4.7	0.691	0.167	0.524	20.9
5.4	1.037	0.167	0.870	34.8
6.0	0.826	0.167	0.659	26.4
6.5	0.351	0.167	0.184	7.4
7.1	0.308	0.167	0.141	5.6
		-Magnesium phytate derivatives***-		
3.6	0.486	0.173	0.313	23.5
4.1	0.610	0.173	0.437	32.9
5.2	0.864	0.173	0.691	52.0
5.9	0.961	0.173	0.788	59.2
6.7	0.616	0.173	0.439	33.0

*Mainly added with enzyme
**Phytate P present, 2.5 mg.; magnesium present, 12.264 mg. (8 ml. of solution)
***Phytate derivative P present, 1.33 mg.; magnesium present, 6.531 mg. (4.26 ml. of solution)

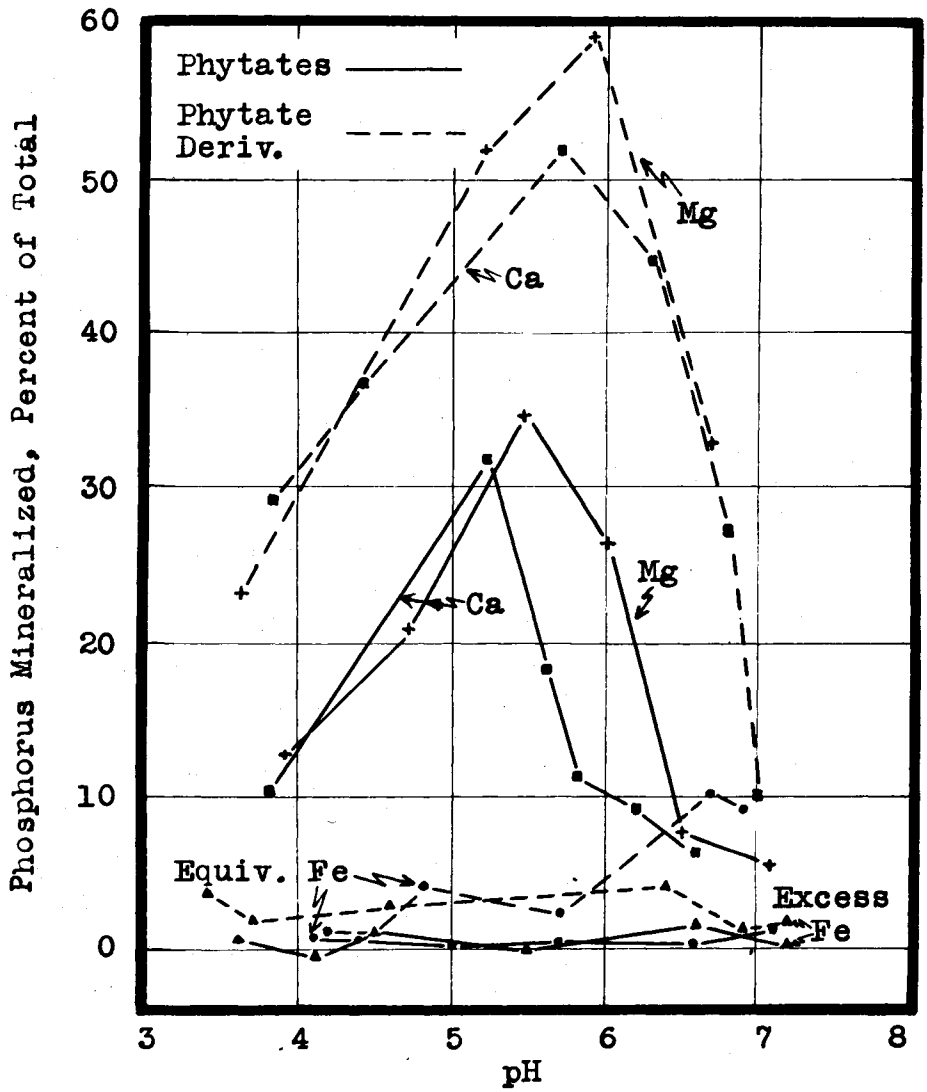

Figure 6. Mineralization at different pH values of ferric, calcium, and magnesium phytates and phytate derivatives by phytase.

The close relationship between the solubilities of the different salts and their degrees of hydrolysis is immediately apparent. The relatively soluble phytate derivatives were hydrolyzed to a much greater extent than were the relatively insoluble phytates. The more soluble calcium and magnesium salts were considerably hydrolyzed, whereas those of iron and aluminum were hydrolyzed only to a very small extent, if at all. It can not be stated with certainty whether there was no hydrolysis of the ferric and aluminum phytates. Since only one analysis was made per sample the errors in analysis were insufficiently taken care of. In any case the hydrolysis was very small.

Gaarder (24) found that ferric hydroxide begins to precipitate at pH 2.4 and is fully precipitated at pH 4, and that aluminum hydroxide begins to precipitate at pH 4 and is fully precipitated at pH 5. In the previous solubility studies, ferric and aluminum phytates were fully insoluble up to pH values of 8 and above. It seems probable that at these higher pH values much of the phytate was adsorbed by the hydroxides or by some type of ferric and aluminum hydroxy phytate compound. It might be expected that these adsorbed phytate ions would be more easily hydrolyzed than would the insoluble salts themselves. The results presented here show that if such adsorption does take place the bonding is so strong that the phytate is inaccessible to the enzyme.

Only in the case of ferric phytate derivatives when the iron was in equivalent amounts was there any definite hydrolysis and this hydrolysis increased with increase in pH, as did the solubility. Where iron was in excess there was apparently some hydrolysis, but the slope of the curve suggests that this result is probably erroneous.

The dependence of hydrolysis on the solubility is well illustrated in the case of the calcium and magnesium phytates at pH values greater than 5.5. Comparisons of the solubility and hydrolysis curves show a strong parallelism.

It is concluded from the above results that in the systems investigated, the rate of hydrolysis of the phytates is limited mainly by the degree of solubility of the phytates. There are some exceptions, however. For instance, at pH 7 the solubility of the calcium phytate derivatives is considerably greater than is that of calcium phytate, yet the degrees of hydrolysis were very similar. Because of the effect of pH on the phytase activity the possible limiting effect of the amount of the phytase becomes more and more probable as the pH differs increasingly from about 5.5. In short term experiments, as in the present case, the effect of the pH on the phytase is marked, but when the hydrolysis is continued for a long time, as must occur in the

soil it is probable that this effect will be of importance only at very high or very low pH values.

Bower's (10) findings that of the inositol bound phosphorus in the soil, only about 30 per cent was as phytate derivatives can be explained by the greater rate of hydrolysis of the phytate derivatives resulting from their greater solubilities. The rate of hydrolysis of phytate derivatives is therefore not the limiting link in determining the rate of change of phytin phosphorus to inorganic phosphorus. For this reason the mineralization studies to be described in the following sections were limited to work with phytate phosphorus.

PHYTASE ACTIVITY IN SOILS.

It was postulated in the introduction that while the effect of pH changes on the availability of phytin phosphorus to plants might be fully explained on the basis of changes in the solubility of the phytin, this varying availability might also be due to changes in the level of phytase activity in the soil as a result of alteration in microbial activity. The present phase of the work is concerned with demonstrating the presence of phytase in soil, relating the level of phytase activity to that of microbial activity and determining the effect of the variation of some soil properties on the phytase activity in soils.

Development of Method of Measuring Phytase Activity in Soil

Since a method of measuring phytase activity in soil did not exist, it was necessary to develop one. It was realized at the outset that the method would necessarily be empirical and would have to be based on the measurement of the mineralization of phytate phosphorus resulting from the incubation under standard conditions of a soluble phytate

in the presence of phytase. Within this framework there were two possibilities, either the extraction of the enzyme from the soil and its subsequent addition to the phytate or the addition of the phytate to the soil.

The former alternative was tried first. Two fresh, moist samples of soil from the field were shaken with water (1:1) and then filtered. Ten ml. of the clear filtrate were added to a solution of sodium phytate buffered to pH 4.9 with acetate and the mixture was incubated for 5 hours at 42° C. The final concentration of phytate phosphorus was 50 p.p.m. Analysis showed no increase in inorganic phosphorus in the solution. It was therefore concluded that if phytase was present it could not be extracted by water.

The alternative procedure of adding phytate phosphorus to the soil was tried using fresh Webster and Clarion soils. Five-g. samples of soil were weighed into Erlenmeyer flasks and a solution of sodium phytate was added to give a final phytate phosphorus concentration of 100 p.p.m. The pH was adjusted to values between 4 and 8 with dilute acid and the mixtures were incubated for 14 hours at 40° C. The samples were then filtered and washed with 25 ml. of 1.3 N hydrochloric acid and the increase in inorganic phosphorus from the added phytate was determined. An increase in inorganic phosphorus was found, indicating the presence of phytase in the soils, but the magnitude of the increase was too small

to permit accurate measurement.

An additional experiment was conducted to determine whether the small mineralization obtained in the above experiment was due to a very low phytase activity in the soil or to whether it might have been due to the fixation of the added phytate. To investigate the latter possibility, sodium phytate was added to 5-g. lots of the above two soils and the pH was adjusted to values between 5 and 7. The final concentrations of the phytate phosphorus were 100 and 550 p.p.m. After 14 hours' shaking, the mixtures were filtered and washed with a little water, and the recovery of the phytate phosphorus was determined in the filtrates. Where the phytate phosphorus concentration was 100 p.p.m. the maximum recovery was 3 per cent while with the concentration of 550 p.p.m. it was 13 per cent. The lower the pH the lower was the recovery. This high fixation could account for the low mineralization previously found.

It is probable that at lower pH values the added sodium phytate was precipitated as the iron and/or aluminum salts. It is well known that various hydroxy organic acids form soluble complexes with iron and aluminum. Of these, citric acid is very effective. Hoff-Jørgensen and Porsdal (33) found that citrate also activates rye bran phytase irrespective of any action it may have on the substrate. Such an activation would be an advantage in the present case

since the quantities to be measured would be larger and more accurately determined. The effect of citrate on the solubility of sodium phytate added to soil was therefore investigated. Varying amounts of potassium citrate were added to 5-g. samples of Clarion soil together with sodium phytate such that the final concentration of phytate phosphorus was approximately 115 p.p.m. The pH was adjusted to approximately 5, 6 and 7 with dilute hydrochloric acid. A parallel series was run with the phytate replaced with an equal volume of water. The total volume of reagents was 20 ml. Both series were incubated together for 12 hours at 50° C and then filtered and washed with a little water. Organic phosphorus was determined in the filtrate. The results are given in Table 18.

Since the recovery of the phytate phosphorus was found by difference an error was introduced because the pH values within the pairs are not identical. The error is small, however, and does not mask the general increase in solubility of the added sodium phytate with increase in citrate concentration and with lowering of pH. At pH 5.2 and with a citrate concentration of 61 per cent the added sodium phytate was practically fully soluble. The inorganic phosphorus measured in the filtrate was always very small and the quantities paralled those of the recovered phytate phosphorus, being greater the lower the pH and the greater the

Table 18. Recovery of phytate phosphorus added to Clarion silt loam in the presence of different amounts of potassium citrate and at different pH values.

Phytate phosphorus added, mg.	Potassium citrate concentration, per cent salt in solution	Final pH	Organic phosphorus in filtrate mg.	Recovery of added phytate phosphorus, Milligrams	Per cent
2.33	0	5.1	0.10	-	-
2.33	0	6.2	0.07	-	-
2.33	0	7.2	0.08	-	-
0	3.05	7.3	0.09		
2.33	3.05	5.4	1.36	1.27	54.5
0	3.05	7.6	0.11		
2.33	3.05	6.4	1.18	1.07	45.9
0	3.05	8.0	0.15		
2.33	3.05	7.5	0.63	0.48	20.6
0	15.25	5.8	0.21		
2.33	15.25	5.1	2.15	1.94	83.2
0	15.25	6.7	0.14		
2.33	15.25	6.3	1.89	1.75	75.1
0	15.25	7.5	0.12		
2.33	15.25	7.3	1.52	1.40	60.1
0	30.50	5.9	0.25		
2.33	30.50	5.1	2.35	2.10	90.1
0	30.50	6.6	0.20		
2.33	30.50	6.2	2.12	1.92	82.4
0	30.50	7.4	0.16		
2.33	30.50	7.1	1.77	1.61	69.1
0	61.00	5.9	0.30		
2.33	61.00	5.2	2.58	2.28	97.8
0	61.00	6.5	0.24		
2.33	61.00	6.1	2.39	2.15	92.2
0	61.00	7.3	0.19		
2.33	61.00	7.2	1.94	1.75	75.1

citrate concentration.

Hoff-Jørgensen and Porsdal (33) found activation of bran phytase by citric acid when used in concentrations of 1 per cent. Since the high concentrations of citrate used above might inactivate the phytase the effect of citrate concentration on phytase activity was investigated. Increasing amounts of potassium citrate were added to known amounts of a sodium phytate solution and the reaction adjusted to pH 5 with hydrochloric acid. The final volume was 20 ml. and the phytate phosphorus concentration was 102 p.p.m. Two ml. of an 0.2 per cent suspension of phytase extract (prepared as previously described) were added, and the increase in inorganic phosphorus was measured after the mixtures had been incubated for 1.75 hours at 45° C.

The results are given in Table 19 and in Figure 7. Included in Figure 7 is the percentage solubility of sodium phytate added to soil adjusted to pH 5. The latter data were obtained by extrapolation of the data of Table 18.

The maximum activation of the phytase was found to be at about 3 per cent citrate concentration which agrees fairly well with Hoff-Jørgensen and Porsdal's findings. However at citrate concentrations greater than 7 per cent the activity of the phytase was progressively reduced but the solubility of the phytate continued to increase. For the present purpose it was necessary to select some arbi-

Table 19. Mineralization of phytate phosphorus by wheat bran phytase in solutions of different potassium citrate concentration.

Phytate phosphorus concentration, p.p.m.	Potassium citrate concentration, per cent of salt in solution	Inorganic phosphorus measured, p.p.m.	Phytate phosphorus mineralized, p.p.m.
0	0	10.8	-
102.5	0	14.5	3.7
102.5	1.0	19.6	8.8
102.5	5.0	19.6	8.8
102.5	15.0	14.9	4.1
102.5	32.5	11.6	0.8

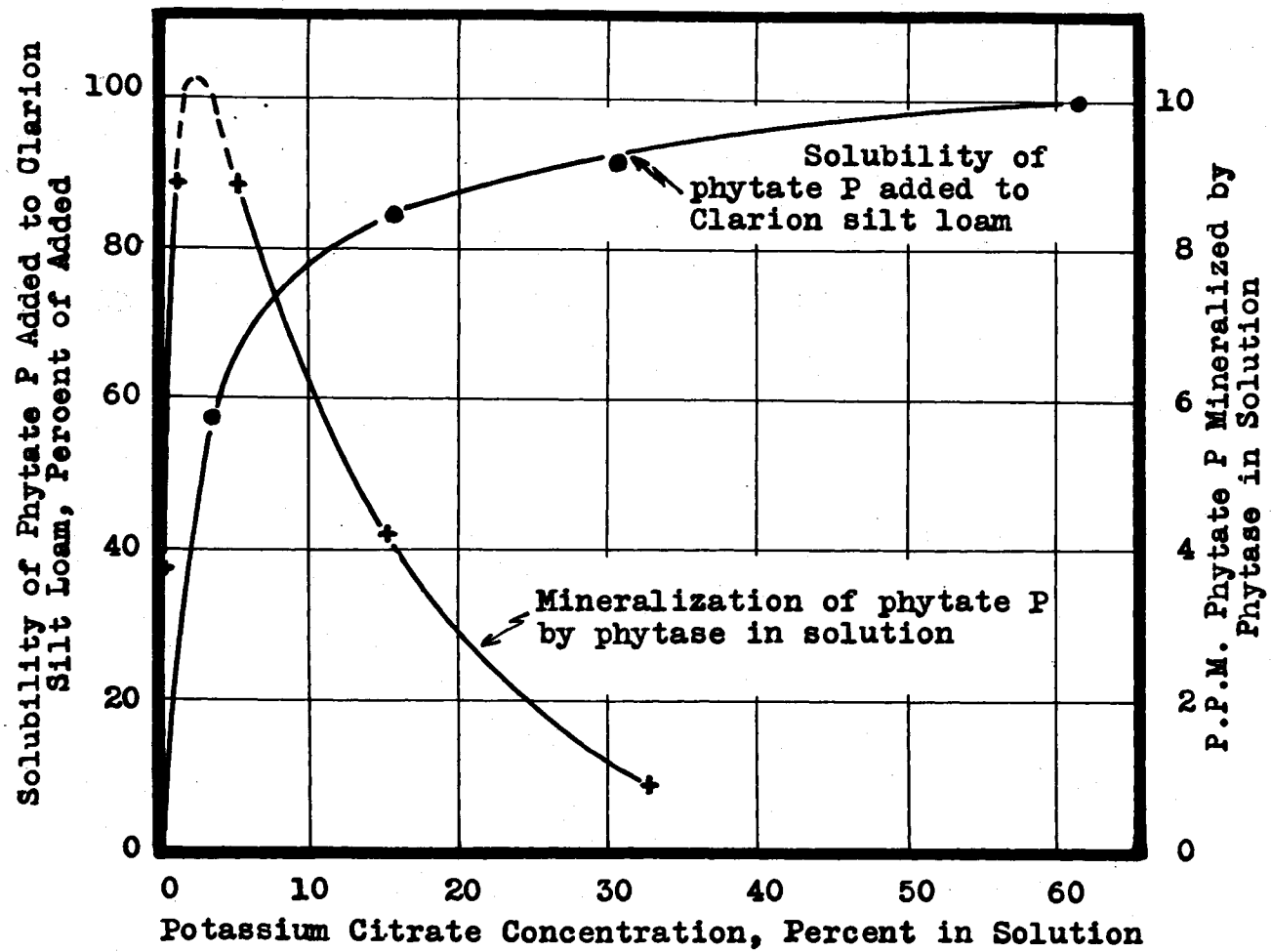

Figure 7. Solubility of phytate phosphorus added to Clarion silt loam and mineralization of phytate phosphorus in solution by wheat bran phytase.

trary citrate concentration such that a high solubility of the phytate would be obtained and at the same time the phytase would be activated.

A concentration of 5 per cent of potassium citrate was selected as the basis for future work since higher concentrations would give relatively small increases in solubility but large reductions in phytase activity. At this concentration about 70 per cent of the sodium phytate was soluble and the activation factor for the phytase was between 2 and 3. The 70 per cent solubility of the sodium phytate will vary with soils of differing fixing capacity. Since the rate of mineralization of the phytate phosphorus depends on its concentration in solution and since this in turn depends on the extent of fixation, comparisons between soils would not be valid if the degree of phytate fixation varied. No estimates of the magnitudes of such variations were made when the citrate concentration was 5 per cent. However such an estimate was made in a previous experiment using widely different soils and with a citrate concentration of 32 per cent. Potassium citrate and sodium phytate were added to 5-g. samples of the dry soils and the pH was adjusted to 5 with dilute hydrochloric acid. The final volume of the reagents added was 20 ml. and the final concentration of the phytate phosphorus was 102 p.p.m. After 4 hours' shaking at 50° C the samples were filtered and washed with a little

water and the increase in the organic phosphorus in the filtrates was determined. The results are given in Table 20.

Table 20. Recovery of phytate phosphorus added to various soils with a 32 per cent concentration of potassium citrate and a pH value of 5.

Soil number	Soil type	Great soil group	Original pH of soil	Recovery of added phytate phosphorus, per cent
3199	Fayette silt loam	Gray-brown podzolic	5.6	95
3198	Cecil sand loam	Red podzolic	5.7	92
3201	Carrington silt loam	Prairie	4.9	89
-	Clarion silt loam	Prairie	<6.5	93
3206	Clarion silt loam*	Prairie	7.5	82
3204	Monona-Ida silt loam transition	Lithosol	7.4	38

*From Figure 7

With the four acid soils the recovery of phytate phosphorus was relatively constant despite the low concentration used. Both the Clarion of pH 7.5 (taken from a road cut) and the Monona-Ida soils had some free calcium carbonate, though not in great amount, and both of these soils showed low recoveries. It is possible that the lowered recoveries in the case of these two soils resulted from a rise in pH due to the free carbonate. No pH determinations were made at the end of the experiment. These results from systems having 32 per cent citrate cannot be strictly applied to systems having 5 per cent citrate. However the main effect of

citrate in increasing phytate solubility occurs at concentrations less than 5 per cent and it is probable that with soils free of calcium carbonate the variations in solubility at this concentration are not great. They may still be significant however. These variations can be reduced relative to the concentration of soluble phytate by greatly increasing the amount of phytate added.

The use of high phytate concentrations has theoretical justification based on Michaelis' (48) theory of enzyme action. Using the hypothesis that the enzyme forms an unstable intermediary compound with the substrate he showed that the velocity of an enzyme catalyzed change is proportional to the enzyme concentration and independent of the substrate concentration if this latter is sufficiently high. Increasing the substrate concentration appeared to be the best way of minimizing the effect of variable fixation and accordingly the mineralization of phytate phosphorus added to soil in higher concentrations was investigated.

Clarion silt loam, to which a little sugar had been added, was moistened and incubated to develop microbial activity. The soil was then partially dried, passed through a 1-mm. sieve and well mixed. Five-gram samples were weighed into a series of 250-ml. Erlenmeyer flasks. Five ml. of 20 per cent potassium citrate previously titrated to pH 5 with hydrochloric acid were then added, followed

by increasing amounts of a sodium phytate solution, also previously titrated to pH 5. Water was added to make the final volume of reagents to 20 ml. A few drops of toluene were added, and the flasks were tightly stoppered and incubated for 20 hours at 35, 45, and 55° C. The different temperatures were used to determine which gave the greater mineralization. The samples were then filtered on Büchner funnels and washed with 100 ml. of 2 N hydrochloric acid. The filtrates were analyzed for inorganic phosphorus. A reaction of pH 5 was selected without further investigation since data in the literature show that this is approximately the optimum pH for phytase. The results are given in Table 21 and Figure 8.

The results show that the mineralization was not fully independent of the phytate concentration even at the highest level used. The slopes of the curves at 35 and 45° C are practically constant when the amount of phytate phosphorus added is greater than about 40 mg. and at the higher values there is little decrease in the dependence of the mineralization on the phytate phosphorus concentration. Sixty mg. was selected as a quantity of phytate phosphorus adequate for the present purpose. From Table 20 it can be seen that in the case of the four acid soils and with a phytate phosphorus concentration of 102 p.p.m. the average recovery was 92.5 per cent with the maximum deviation being 3.5 per cent.

Table 21. The mineralization of phytate phosphorus added in increasing amounts to phytase-active Clarion silt loam and incubated at three temperatures.

Incubation temperature, ° C	Phytate phosphorus added, mg.	Inorganic phosphorus in filtrate, mg.	Inorganic phosphorus added with phytate, mg.	Phytate phosphorus mineralized, mg.
35° C	0.00	0.25	-	-
	23.48	1.10	0.24	0.61
	46.96	1.55	0.47	0.83
	71.44	1.92	0.71	0.96
	93.92	2.24	0.94	1.05
45° C	0.00	0.27	-	-
	23.48	1.36	0.24	0.85
	46.96	1.78	0.47	1.04
	71.44	2.18	0.71	1.20
	93.92	2.55	0.94	1.34
55° C	0.00	0.25	-	-
	23.48	1.14	0.24	0.65
	46.96	1.72	0.47	1.00
	71.44	2.10	0.71	1.14
	93.92	2.80	0.94	1.61

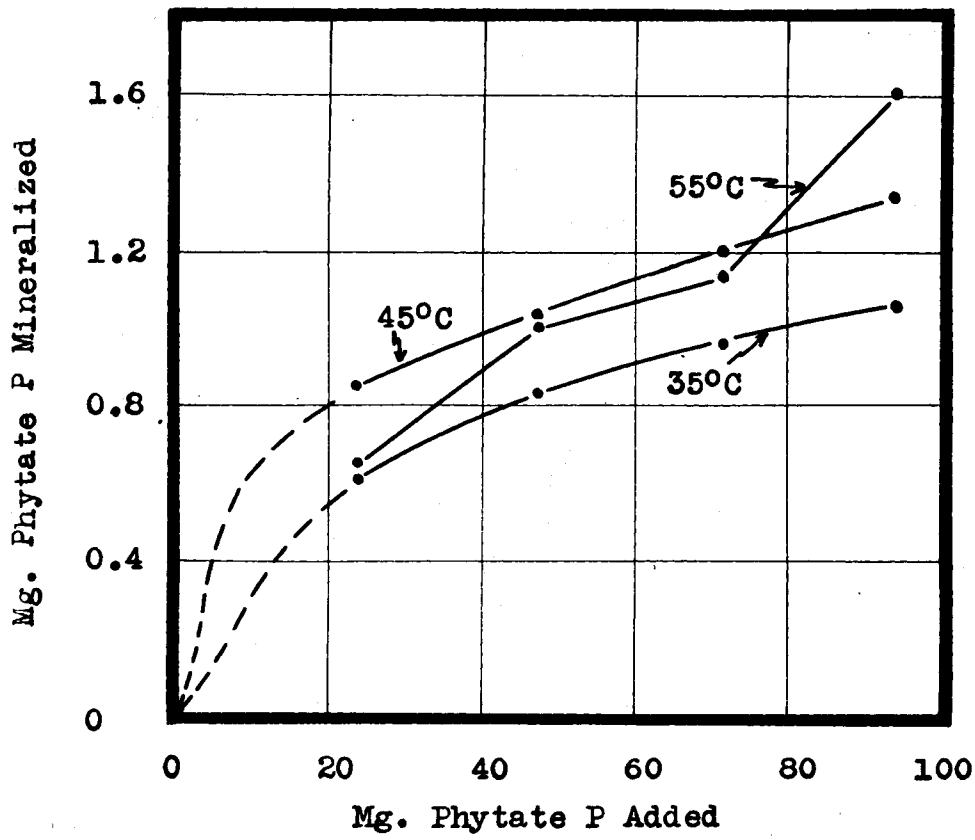

Figure 8. The mineralization of phytate phosphorus when added in increasing amounts to phytase-active Clarion silt loam and incubated at three temperatures.

With 60 mg. phytate phosphorus added (3000 p.p.m.) the deviations should be small. Even with variations in fixation as great as 10 per cent the resulting error would be less than 5 per cent, a value that was considered to be within the limits allowable for such a method. Furthermore, the phytase activity level in the samples used was probably higher than would be found in field soils, due to the artificially high microbial activity. With lowered activities the corresponding curves would have progressively decreasing slopes at corresponding points and the error due to variable fixation would be reduced.

Mineralization was greatest at 45° C and this incubation temperature was selected in preference to 35 or 55° C. Although temperature optima reported in the literature range from 50 to 55° C there is no necessary contradiction since, as Baldwin (5) points out, the optimum temperature is inversely related to the time of incubation.

In the previous experiments with Clarion soil the mineralized phosphorus was extracted by filtering and washing with varying amounts of from 1 to 2 N hydrochloric acid. It was not known if such extractants would be effective with other soils. Accordingly, various concentrations and amounts of hydrochloric acid were tried with several other soils to which had been added known amounts

of potassium dihydrogen phosphate. The amount of soil, potassium citrate concentration and pH adjustment were as in the previous experiment. The conditions of incubation, extractants used, and results are given in Table 22.

The recoveries were low where 2 N hydrochloric acid was used but with 100 ml. of 4 N acid they were nearly quantitative though rather low in the case of the acid Carrington soil. The method finally selected, which was that used with the Marion and Shelby soils, was to add 50 ml. of 4 N hydrochloric acid to the flasks at the end of incubation, to allow them to stand for 30 minutes and then to filter them on Buchner funnels and wash with 100 ml. of 4 N hydrochloric acid. This method is not universally successful, as is shown in the case of the Shelby subsoil. This subsoil, when further digested in hot 0.5 N sodium hydroxide, gave a total recovery of 100.5 per cent.

The inorganic phosphorus was determined by the method of Dickman and Bray (17). Despite the large amount of iron that is extracted the method is satisfactory. There is fading of the blue color due to the iron present but these errors cancel out if all readings are taken at a constant time after the addition of the stannous chloride. The data in Table 23, obtained by taking readings after increasing intervals of time after the development of the blue color, confirm the validity of the method.

Table 22. The recovery from various soils of added inorganic phosphorus upon extraction with different concentrations and amounts of hydrochloric acid.

Soil type	Inorganic phosphorus added, mg.	Extractant	Inorganic phosphorus in extract, mg.	Recovery of added phosphorus per cent
Cecil sandy loam*	0	2 N HCl, 100 ml.	0.19	
	0.404	2 N HCl, 100 ml.	0.56	92
	0	2 N HCl, 150 ml.	0.26	
	0.404	2 N HCl, 150 ml.	0.586	92
	0	4 N HCl, 100 ml.	0.25	
	0.404	4 N HCl, 100 ml.	0.65	99
Carrington silt loam*	0	2 N HCl, 100 ml.	0.246	
	0.404	2 N HCl, 100 ml.	0.586	84
	0	2 N HCl, 150 ml.	0.296	
	0.404	2 N HCl, 150 ml.	0.646	87
	0	4 N HCl, 100 ml.	0.32	
	0.404	4 N HCl, 100 ml.	0.70	94
Fayette silt loam*	0	2 N HCl, 100 ml.	0.804	
	0.404	2 N HCl, 100 ml.	1.166	90
	0	2 N HCl, 150 ml.	0.884	
	0.404	2 N HCl, 150 ml.	1.256	92
	0	4 N HCl, 100 ml.	0.97	
	0.404	4 N HCl, 100 ml.	1.38	101
Marion silt loam**	0	4 N HCl, 150 ml.	0.68	
	4.0	4 N HCl, 150 ml.	4.58	98
Shelby silt loam, C horizon**	0	4 N HCl, 150 ml.	0.24	
	4.0	4 N HCl, 150 ml.	3.30	77

*Incubated for 20 hours at 45° C
**Incubated for 4 days at room temperature

Table 23. Determination of inorganic phosphorus in 4 N hydrochloric acid extracts of soils using the method of Dickman and Bray taking the final reading at increasing time intervals after adding the stannous chloride.

System	Inorganic phosphorus added, p.p.m.	Time of development of color, minutes	Inorganic phosphorus measured, p.p.m.*	Recovery of added phosphorus	
				p.p.m.	per cent
Fayette silt loam					
Extract alone	-	10	0.028	-	-
Extract plus P	0.187	10	0.396	0.188	100.5
Extract alone	-	17	0.204	-	-
Extract plus P	0.187	17	0.388	0.184	98.5
Cecil sandy loam					
Extract alone	-	5	0.112	-	-
Extract plus P	0.187	5	0.299	0.187	100.0
Extract alone	-	13	0.107	-	-
Extract plus P	0.187	13	0.293	0.186	99.5
Extract alone	-	17	0.105	-	-
Extract plus P	0.187	17	0.291	0.186	99.5

*Measured using a calibration curve made from standard iron-free phosphorus solutions

Method of Measuring Phytase Activity in Soil

The method which was finally used and which was based on the preceding results was as follows. Five-g. samples of the well mixed soil were weighed into two 250 ml. Erlenmeyer flasks. Ten ml. of the potassium citrate solution* followed by 10 ml. of the sodium phytate solution** were measured into one flask. The same amount of citrate was added to the other flask but the sodium phytate was replaced by 10 ml. of water. Ten drops of toluene were then added to both flasks which were then stoppered securely. The contents were gently mixed so that as little as possible was left on the sides of the flasks and then incubated for 20 hours at 45° C. At the end of this time 50 ml. of 4 N hydrochloric acid were added and the flasks were allowed to stand for 30 minutes. The mixtures were then filtered on Büchner funnels to which a little fine asbestos had been added to prevent leakage of the soil particles around the edge of the paper. The samples were washed with 100 ml. of 4 N

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* A 10 per cent solution. Ten g. of potassium citrate monohydrate were dissolved in water and titrated to pH 5 with hydrochloric acid. The solution was then diluted to 100 ml.

**A solution containing 6000 p.p.m. phytate phosphorus and titrated to pH 5 with hydrochloric acid. The inorganic phosphorus content was determined and the solution was kept in the refrigerator to eliminate the development of molds.

hydrochloric acid in quantities of 20 ml., allowing each fraction to filter through before the next was added. If the analyses of the filtrates were to be done the same day, the filtrates were diluted to 500 ml. and the inorganic phosphorus was determined by the method of Dickman and Bray (17), taking the final reading 10 minutes after the color had been developed by the addition of stannous chloride. If the analyses were to be delayed, the filtrates were neutralized with concentrated ammonia and cooled in water. The ammonia precipitates hydroxides, phosphates and phytates. Before analysis, hydrochloric acid was added so as to just dissolve these precipitates after which the solutions were made to volume and analyzed as before. The difference between the phosphorus measured with sodium phytate present and that with no sodium phytate present was taken as the measure of the phytase activity.

An estimate of the precision of the method was made by making 19 determinations on a sample of thoroughly mixed Clarion soil. Two colorimetric determinations were made on each sample. The data were analyzed statistically with the following results, all expressed in concentration (p.p.m.) of mineralized phytin phosphorus in the extract:

Mean.....10.4 p.p.m.

Range..... 9.7 to 11.7 p.p.m.

Standard deviation.... 0.63 p.p.m.

Subsequent use of the method indicates that the precision may be rather better than indicated here, although no further estimates were made.

In a previous experiment similar to that reported above, the necessity of accurate temperature control during incubation was noted. The samples were divided between two different shelves in the oven. It was found that the shelf temperatures differed by 2° C. When this temperature effect was included in the statistical analysis it was found that the difference in temperature had caused a significant difference in mineralization.

Effect of Storage and Drying on the Phytase Activity of Soils

Enzymes, which are essentially proteins, are relatively unstable as regards their ability to act as catalysts. Ensminger and Gieseking (20) have shown that proteins are adsorbed by clays and Bower (11) found that if a solution containing nuclease was shaken with montmorillonite the nuclease activity was reduced. Phytase appears to be produced in the soil by micro-organisms. Thus the activity as measured at any one time is the resultant of the production of the enzyme and the deactivation by clays, proteolytic enzymes or other agencies. Since drying might be expected

to deactivate the enzyme and since differences in length of storage might change the activity of the microbial population these two factors were investigated. The main objective was to determine what treatment was necessary prior to the phytase determination such that the results would be comparable.

Three soils of different textures were incubated moist and with a little sugar and nitrogen added. They were then thoroughly mixed and portions were dried to varying degrees using a fan at room temperature. The moisture content and the phytase activity were then determined. One soil was kept stored in 2-ounce, screw-capped medicine bottles at room temperature and the activity was determined after increasing periods of time.

The results are given in Table 24 and those for the activities at zero time are given in Figure 9. These results show that both drying and storage affect the activity considerably. The more extreme the drying the lower is the activity. The smaller the drying the greater is the variation due to storage. This change on storage was undoubtedly due to microbial synthesis of new phytase. With increasing time of storage the energy supply was evidently depleted and with this depletion there was decreased microbial activity and a resulting decreased synthesis of phytase.

Table 24. The phytase activity of soils of different textures having different moisture contents and after storage for increasing periods of time.

Soil texture	Soil moisture, per cent*	Phytase activity (mg. P released) after indicated time of storage, hours**				
		0	25	68	164	332
Silt loam	13.2	1.54 (1.78)	2.07 (2.39)	2.35 (2.71)	2.14 (2.47)	1.89 (2.18)
	9.2	1.43 (1.57)	1.58 (1.74)	1.85 (2.04)	1.87 (2.06)	1.71 (1.88)
	2.0	0.83 (0.85)	0.86 (0.88)	0.89 (0.91)	0.81 (0.83)	0.72 (0.73)
Clay loam	23.2	3.40 (4.43)				
	19.4	3.43 (4.31)				
	12.4	3.59 (4.10)				
	9.1	3.30 (3.62)				
	3.8	2.46 (2.56)				
Fine sandy loam	16.5	7.23 (8.65)				
	11.0	6.25 (7.02)				
	6.7	5.85 (6.27)				
	3.4	5.57 (5.78)				
	1.4	4.51 (4.57)				

*On oven-dry (105° C) basis

**Unbracketed figures are on the basis of the 5 grams of moist soil used. The bracketed figures are on the basis of 5 grams of oven-dry soil.

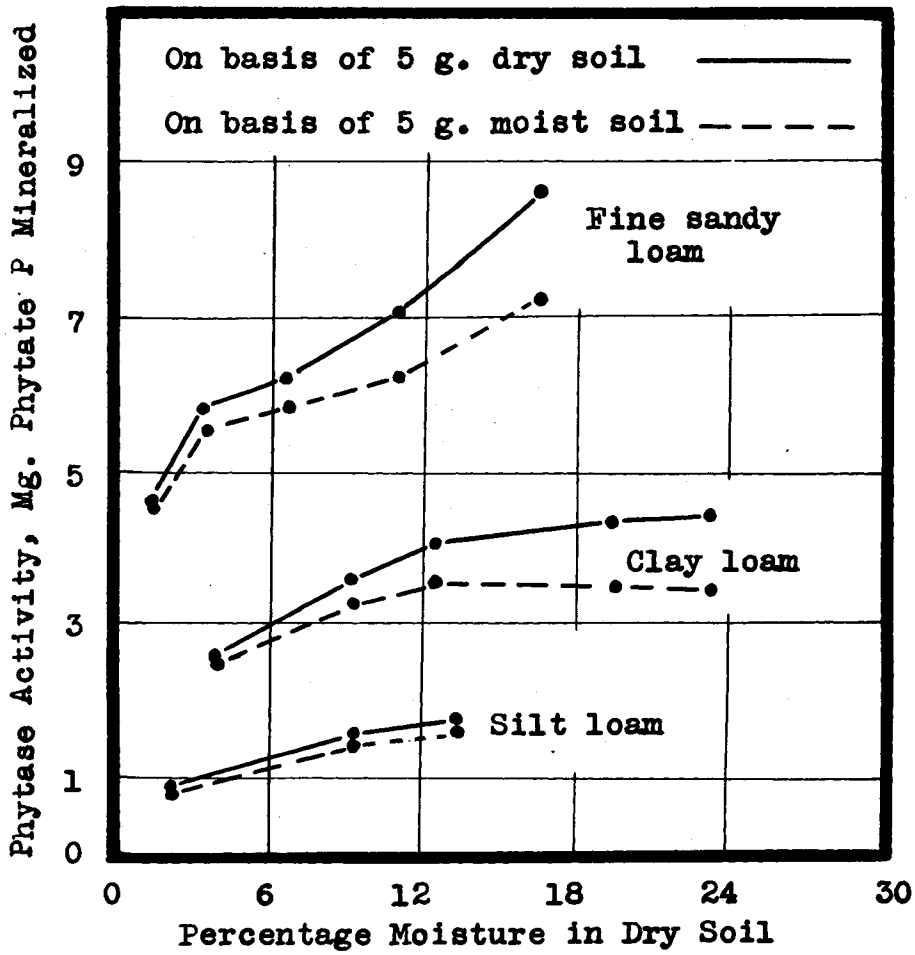

Figure 9. Phytase activity of three soils of different textures dried to varying degrees prior to analysis.

The soils used had very high and unusually active microbial populations due to the energy material added. In field soils the supply of readily available energy material will be less than in the present case and very probably the fluctuations, especially those due to storage, will not be so great. Nevertheless these findings show that the method of determining phytase activity is one of limited application. When comparing the activities of different soil samples their previous histories must be taken into account. The method is of greatest value in comparative work involving one soil. The results also show that once a soil has been sampled, the determination of phytase activity should be made as quickly as possible and the soil should be handled as little as possible consistent with obtaining a representative sample.

The decrease in activity as a result of drying may be caused by denaturation of the enzyme due to dehydration. It may also be due to increased adsorption by soil colloids resulting from the more intimate contact of the enzyme with these colloids as the moisture is progressively reduced. Figure 9 supports this second explanation. While the curves are far from complete, they show similarities to adsorption isotherms, especially in the case of the clay loam which has the highest colloid content of the three

soils. This explanation is further supported by the results of the following experiment.

Three series of 5-g. samples of phytase-active Clarion silt loam were set up. The volume of reagents added (20 ml.), the reaction (pH 5), the potassium citrate concentration (5 per cent), and the increasing amounts of sodium phytate solution added were all the same for the three series. A constant amount of a bran phytase extract was added to all the samples. The only differences in treatment were in the order and method of adding the phytase extract and in the method of incubation. These differences are detailed below.

Series 1. The enzyme was added last. Incubation was for 10 hours at 45° C and during incubation the flasks were continuously rotated.

Series 2. The enzyme was added as in 1. Incubation was for 14 hours at 45° C but the flasks were shaken only occasionally.

Series 3. The enzyme was added to the dry soil which was then dried by fanning at room temperature until the color began to lighten. The other reagents were then added. Incubation was as in 2.

At the end of the incubation the mineralization of the phytate phosphorus was determined. The results are given in Table 25 and Figure 10. The curves of series 1 and 3

Table 25. The mineralization, by wheat bran phytase, of phytate phosphorus added to Clarion silt loam as affected by different methods of adding the phytase and different methods of incubation.

Phytate phosphorus added, mg.	Total inorganic phosphorus measured, mg.	Inorganic phosphorus added with phytate, mg.	Phytate phosphorus mineralized, mg.
		- Series 1 -	
0	0.40	0	-
1.02	0.54	0.01	0.13
2.04	0.54	0.02	0.12
3.06	0.54	0.03	0.11
11.74	0.72	0.11	0.21
23.48	0.84	0.23	0.21
46.96	1.10	0.46	0.24
		- Series 2 -	
0	0.54	0	-
2.04	1.12	0.02	0.56
11.74	1.56	0.11	0.91
23.48	1.84	0.23	1.07
46.96	2.28	0.46	1.28
		- Series 3 -	
0	0.38	0	-
2.04	0.51	0.02	0.11
11.74	0.59	0.11	0.10
23.48	0.80	0.23	0.19
46.96	1.02	0.46	0.18

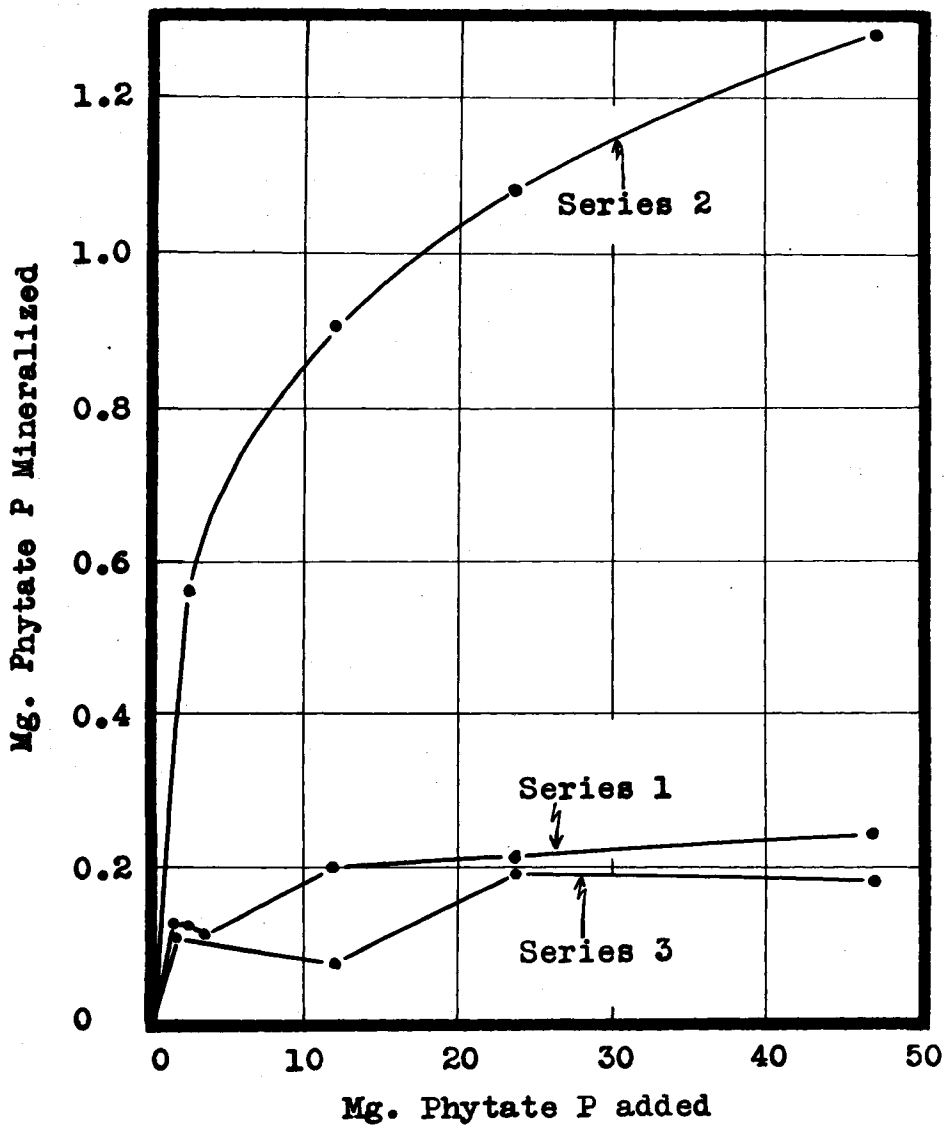

Figure 10. Mineralization of phytate phosphorus added to Clarion silt loam with different conditions of phytase addition and incubation.

are very similar, but are in sharp contrast to that of series 2, where the mineralization was considerably greater. The two former series have the common feature that the added enzyme was able to make greater contact with the soil than it was in series 2. The similarity of the curves of series 1 and 3 indicates that dehydration of the enzyme did not play an important part in reducing phytase activity. Comparison between series 2 and series 1 and 3 indicates that the reduction in activity was due to the adsorption of the phytase by the soil. The large mineralization in series 2 probably took place in the supernatant liquid above the soil since on standing the soil flocculated and settled out.

Microbial Activity and Phytase Activity in Soils

It has been shown above that phytase activity is produced when energy material is added to a soil and the mixture is incubated. Although it was considered probable that the level of this phytase activity was related to the level of the overall physiological activity of the soil population, the quantitative nature of this relationship was unknown.

The overall activity is commonly measured by determining the carbon dioxide produced by microbial respiration. Such data must be interpreted with caution however since

changes in carbon dioxide production can be the result of both qualitative and quantitative changes in the soil population. In mixed cultures the qualitative changes may be very important. If these points are kept in mind the measurement of the carbon dioxide produced still appears to be the best available method of estimating the overall microbial activity of the soil.

Studies were carried out on the relationship between the amount of carbon dioxide produced and the level of phytase activity developed by mixed soil floras of different overall activity. Similar determinations were made using pure and mixed cultures of various bacteria and fungi.

In general the method used was as follows. One hundred g. of Clarion silt loam of pH 5.8 were placed in 500 ml. Erlenmeyer flasks and moistened to varying degrees. Various amendments were added and the mixtures were then steam-sterilized. After the flasks had cooled they were inoculated and placed in a constant temperature (28° C) incubation cabinet. During incubation, sterile, moist, carbon dioxide-free air was continuously passed through the flasks and then into sodium hydroxide tubes where the carbon dioxide was collected and determined by titration in the presence of barium chloride. After 70 hours' incubation, when the rate of carbon dioxide production had appreciably decreased the samples were emptied out and

mixed and the phytase activity determined.

Three separate series were studied. In Series 1, the inoculant was 1 ml. of a water extract of a fresh soil, the moisture content of the samples was 25 per cent of the dry soil weight and the amendment was alfalfa meal added in increasing amounts up to 2 per cent of the soil weight. The phytase activity was determined on the samples as they came from the flasks without any pretreatment. Series 2 was the same but the samples were inoculated with pure and mixed cultures of various fungi and the amendment was 2 per cent of the soil weight of alfalfa meal in all cases. The phytase activity was determined as in Series 1. In Series 3, the samples were inoculated with various species of bacteria.* Moisture content, amendments and time of incubation varied within the series. All were removed from the incubation cabinet on the same date. Prior to the determination of the phytase these samples were partially dried by fanning them at room temperature until the color of the soil started to lighten.

The results for Series 1 are given in Table 26 and Figure 11 and the results for Series 2 are given in Table 27. The characteristics of the samples used and the results for Series 3 are given in Table 28.

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*This portion of the work was performed with the advice and assistance of Dr. Francis E. Clark

Table 26. Series 1. Carbon dioxide produced and phytase activity developed by a mixed microbial population during 5 days' incubation of Clarion silt loam with increasing amounts of alfalfa meal.

Alfalfa meal added, g.	Carbon dioxide produced, mg.	Phytase activity, mg. phytate P mineralized
0*	39	0.07
0	45	0.02
0.4	172	0.14
0.4	161	0.17
0.8	274	0.34
0.8	194	0.34
1.2	383	0.41
1.2	364	0.27
1.6	493	0.50
1.6	452	0.49
2.0	525	0.41
2.0	527	0.54

*Incubation replicate pairs

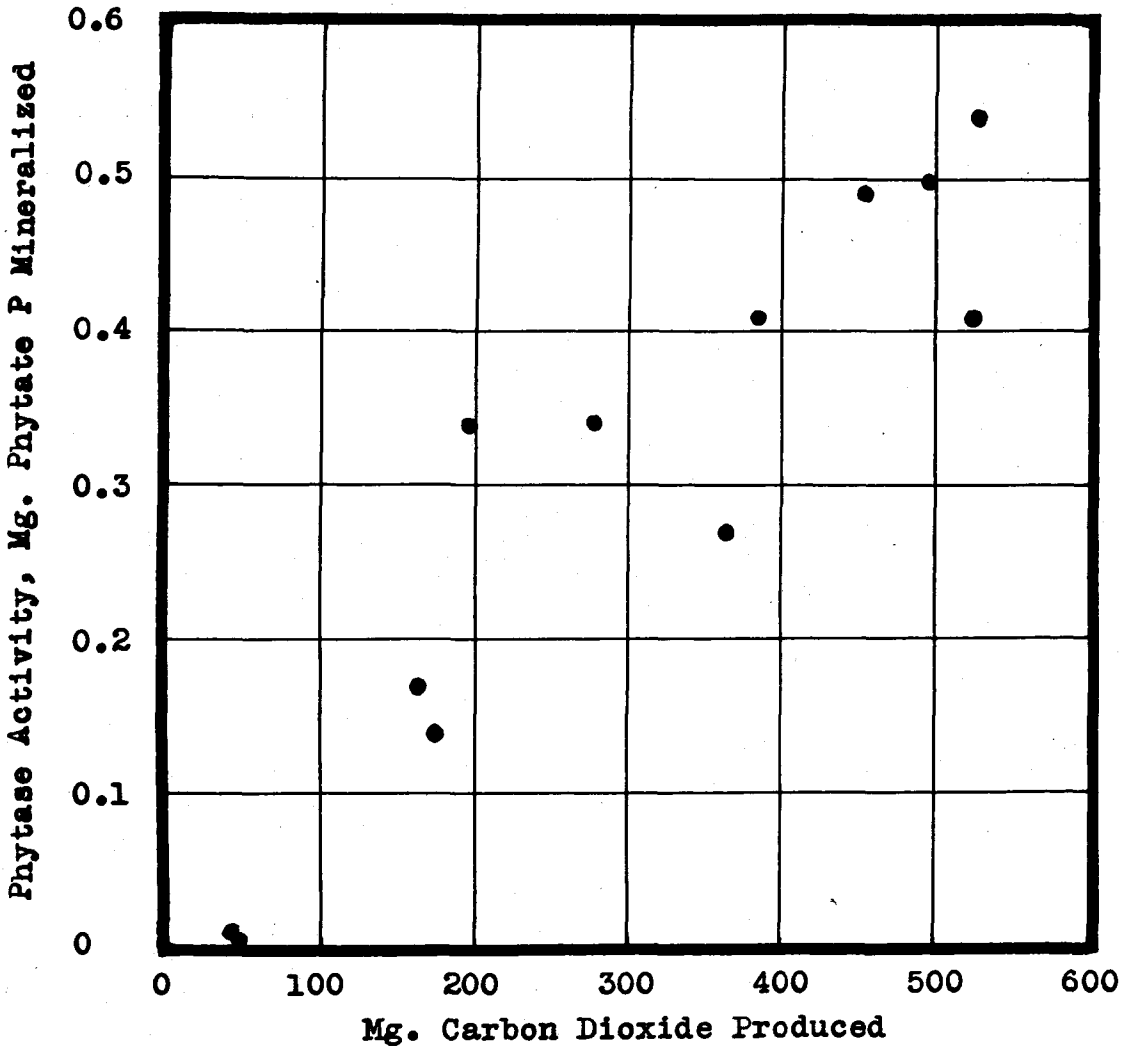

Figure 11. Carbon dioxide production and phytase activity in Clarion silt loam incubated with increasing amounts of alfalfa meal.

Table 27. Series 2. Carbon dioxide produced and phytase activity developed by different fungi during 6 days' incubation of soil containing 2 per cent alfalfa.

Inoculum	Carbon dioxide produced, mg.	Phytase activity, mg. phytate P mineralized*
Aspergillus niger	440	1.74
Penicillium chrysogenum	355	3.11
Cunninghamella blakesleana	313	1.34
Rhizopus nigricans	139	0.095
Actinomucor sp.	191	0.035
Mixture of above fungi (*A. niger* dominant)	456	1.56
Non-sterile soil	750	0.84

*Averages of 2 determinations

Table 28. Series 3. Carbon dioxide produced and phytase activity developed by different bacteria during incubation of soil with different plant materials.

Inoculum	Organic matter added and moisture present	Incubation time, days	Carbon dioxide produced, mg.	Phytase activity, mg. phytate P mineralized
Arthrobacter helvolum (Remained sterile)	1 per cent corn stover; 31.7 per cent moisture	18	5	-0.03
*Arthrobacter** *helvolum*		18	30	0.14
B.cereus	1 per cent alfalfa meal; 20.1 per cent moisture	10	55	0.15
B.subtilis		10	175	0.03
Bacillus sp. (Contaminated last 5 days)		10	400	1.76

*Count at end of incubation, 10^9 organisms per gram

The results given in Table 26 and Figure 11 show that carbon dioxide production and level of phytase activity are directly related. If it can be considered that carbon dioxide production is an adequate measure of overall microbial activity, it follows that with increasing microbial activity there is a correlated increase in phytase activity. In this series all the phytase activity was of microbial origin and it may be inferred that a major part of the activity in field soils is similarly produced. A small part may come from plant material added to the soil but the activity from this source will decrease with time relatively quickly.

It cannot be stated, from the data of these three series, whether in general bacteria or fungi are the main source of phytase activity in soils. Series 2 and 3 cannot be compared owing to the different conditions of incubation and because the Series 3 samples were partially dried before the phytase activities were determined. This drying undoubtedly decreased the activities. However there are some indications that fungi are greater producers of phytase activity than are bacteria. In Series 3 the contaminated Bacillus sp. sample was dried with the other samples but the activity was much higher. Fungi may have been responsible for this high value. In

Series 2 the sample inoculated with non-sterile soil had a disproportionately low phytase activity which may have been due to the reduction of the fungal population by competing bacteria. This possibility is supported by the results of an experiment where phytase activity was developed in two different soils by incubation at three pH values.

Samples of 200 g. of Marion silt loam and Shelby silt loam (C horizon) were incubated with 1 and 0.1 per cent of sugar and nitrogen, respectively. The moisture was 25 per cent of the dry soil weight. One set was incubated for 11 days. The Shelby sample of this set received a small amount of inorganic phosphorus since this soil is very deficient in available phosphorus. Another set was incubated for 2 days and no phosphorus was added to the Shelby sample. Incubation of both series was stopped at the same time and both were then analyzed for phytase activity. The results are given in Table 29.

Except in the case where no phosphorus was added to the Shelby soil the level of phytase activity increased as the pH decreased. It is usually considered that fungi can develop to a greater extent at low pH values than at high pH values due to reduced bacterial competition at the lower pH values. This qualitative change in population may have been the cause of the increasing activity as

Table 29. The phytase activity developed by incubation of two soils at different pH values and for different lengths of time.

Soil type	pH	Phytase activity, mg. phytate P mineralized	
		Incubated 2 days	Incubated 11 days
Marion silt loam	5	4.98	4.30
	6	1.88	2.65
	7	0.87	0.72
Shelby silt loam (C horizon)	5	0.07	4.89
	6	1.10	2.68
	7	0.58	0.88

the pH was lowered. There was no apparent reason why such differences in pH should affect the method of determination. A check was made of the pH of the samples when all the reagents had been added and it was found that the buffering capacity of the potassium citrate was such that the pH values of the various samples did not vary by more than 0.03 of a pH unit.

The pure culture studies indicate that the synthesis of phytase is common to many species of micro-organisms. Its production by Arthrobacter helvolum is especially interesting. Relatively little is known about Arthrobacter. It is a member of Winogradsky's (70) "autochthonous flora", that is, those members of the soil microflora which are widely distributed in soils but which appear to be very unresponsive to changes in environment and which have a low metabolic rate. If soil conditions become such that the more active members of the soil flora are greatly reduced in numbers, the continued presence of Arthrobacter and other autochthonous species will probably ensure a continuous, if low, supply of phytase.

These results further indicate that phytase activity is present in all field soils. Moreover, the level of the activity may generally be higher at lower pH values. If such is the case, then it appears likely that the lack of response of plants to phytate phosphorus added to acid

soils is not due to insufficient phytase activity.

MINERALIZATION OF PHYTATE PHOSPHORUS IN SOIL.

The results of the preceding work suggest that the insolubility of the phytates rather than the lack of enzyme is the main factor which limits the mineralization of phytate phosphorus in the soil. However, the results are merely suggestive and it was desired to establish the point more fully by appropriate experimentation.

The hypothesis to be tested was that the insolubility of the phytates in acid soils is the factor which limits the mineralization of phytate phosphorus. The methods of experimentally testing this hypothesis were based upon three considerations. The first was that the optimum reaction for phytase activity is at about pH 5 while with increasing pH values the activity is considerably reduced. The second was that as the pH of the soil is lowered the solubility of the phytates should be reduced due to the formation of iron and aluminum salts which are less soluble than the calcium salt which predominates at higher pH values. The third was that with low amounts of phytate added to soils of different pH values, the differences in solubility (and hence in mineralization) of the phytate

would be well developed whereas if large amounts were added the differences in concentration of the soluble phytate would be negligible.

Two general procedures were used. In the first, the soils were selected or adjusted so that the pH values ranged from 5 to 7. Various sources of phytase were used, including fresh soil from the field, soil incubated with additions of energy material, and a preparation of phytase from bran. Increasing amounts of sodium phytate or phytase extract were added and after the systems had been incubated the increase in inorganic phosphorus was determined. In the second procedure soils high in organic phosphorus were adjusted to pH values 5, 6, and 7, and then incubated dry, moist, and moist with energy material and nitrogen added. At the end of incubation the phytate phosphorus content was determined.

Mineralization of Phytate Phosphorus Added to Soil

Four experiments were set up which were based on the first procedure outlined above. The expectation was that when the amount of added phytate was low, the mineralization would be less at the low pH values than at the higher values due to the smaller solubility of the phytate under the more acid conditions. However, when the amount of

added phytate was large it was expected that the mineralization would be greater at the lower pH values since, under these conditions, the concentrations of the soluble phytate would be very similar in all cases and the effect of pH on the phytase would be the determining factor.

Materials and methods.

In the first experiment samples of Marion silt loam were adjusted to pH values of 5, 6, and 7. Fifty-g. samples of the dry soil were titrated with dilute hydrochloric acid or a clear, saturated solution of calcium hydroxide. After standing for two days the samples were filtered, washed with a little water and dried. The pH values were then determined. From the resulting buffer curves the amounts of acid or base necessary to give the desired pH values were estimated by interpolation. Sufficient amounts of soil were then adjusted to the appropriate pH values by the same method.

Phytase activity was developed as described on page 102. At the end of this incubation 10-g. samples of the thoroughly mixed soils were weighed into 2-ounce medicine bottles. To each pH series were added increasing amounts of sodium phytate solutions which had been previously titrated with hydrochloric acid to pH values of 5, 6, and

7. Water was added where necessary to bring the volume of liquid added to 6 ml. in all cases. Ten drops of toluene were added to inhibit bacterial action and the bottles were then tightly capped, shaken by hand and allowed to stand at room temperature for 28 days. At the end of this time the inorganic phosphorus was determined by the same procedures used in the method for determining phytase activity.

In the second experiment fresh samples of Carrington silt loam (pH 5.2), Edina silt loam (pH 5.0), and Clarion silt loam (pH 7.5, taken from a cut for a gravel road) were used. There was no development of phytase activity by incubation. Ten-g. samples of the soils were weighed into series of Erlenmeyer flasks. Increasing amounts of sodium phytate solutions which had previously been titrated to pH 5 and 7 were added to the series. In the case of the Clarion soil the phytate caused a slight drop in pH which was accordingly corrected with dilute sodium hydroxide. Water was added to bring the final volume of liquid added to 20 ml. After the addition of 0.5 ml. of toluene the flasks were stoppered and allowed to stand at room temperature for 3 days. The increase in inorganic phosphorus was then determined as before.

For the third experiment three samples of fresh Carrington silt loam were used. One sample came from an

unlimed plot and had a pH of 4.95. The other two samples came from nearby plots which had received 2.5 and 10.5 tons of lime and had pH values of 5.4 and 6.25 respectively. All treatments of these samples were as in the second experiment except that the pH of the incubated mixtures was maintained at the natural pH of the soils by the addition of dilute acid. Addition of acid was necessary since the sodium phytate solution used was at pH 7.

In the fourth experiment the remainder of the samples of the Marion silt loam adjusted to pH values of 5 and 7 in the first experiment were used. Ten-g. samples were weighed into Erlenmeyer flasks followed by increasing amounts of sodium phytate solutions, also at pH 5 and 7. The volumes of added liquids were made to 14 ml. in all cases and after 0.5 ml. of toluene had been added the flasks were placed in the refrigerator. After 3 days the flasks were removed from the refrigerator; 4 ml. of a phytase-active extract of bran* were added to one series and

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*The extract was prepared according to the following procedure. Wheat bran and water (1:5) were shaken for 1 hour. The liquid was squeezed out through cloth and further debris was removed by centrifugation. Ammonium sulfate was added to the centrifugate until the solution was saturated. The resultant precipitate was centrifuged down and then suspended in water and dialyzed for 12 hours using a Visking bag. The remaining precipitate was filtered off and discarded, and the ammonium sulfate precipitation and dialysis was repeated with the filtrate. The final filtrate was pale brown and had a high phytase

4 ml. of water were added to the other. The flasks were then stoppered and allowed to stand at room temperature for 3 days, during which time they were shaken daily. At the end of incubation inorganic phosphorus was determined as before.

Results and discussion.

The results for the first, second, third, and fourth experiments are given in Tables 30, 31, 32, and 33 and in Figures 12, 13, 14, and 15 respectively. Included are the phytase activities of the soils and the phytase extract used. These determinations were made at the beginning of each incubation.

The experiments generally failed to give results that confirmed the hypothesis or indeed that could be interpreted with any certainty at all. In only the second experiment, where fresh samples of different soils were used (Figure 13), was the expected type of curve obtained and here the

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activity.

The ammonium sulfate precipitation of the phytase is much superior to the alcohol precipitation used previously. Comparison of the two methods showed that when the ammonium sulfate was used the activity was about ten times that obtained when alcohol was used under comparable conditions. Dialysis does not reduce the activity. Additional advantages of the present method are that the enzyme is in solution rather than as a suspended solid and that after dialysis there is practically no inorganic phosphorus present in the solution.

Table 30. Mineralization of phytate phosphorus incubated with Marion silt loam of pH 5, 6, and 7.

Phytate phosphorus added, mg.	Phytate phosphorus mineralized at indicated pH values, mg.*		
	pH 5	pH 6	pH 7
1	0.01	0.04	-0.001
2	0.05	0.07	0.12
4	0.32	0.29	0.18
6	0.62	0.46	0.35
8	1.01	0.76	0.63
10	1.41	1.18	0.94
12	2.21	1.63	1.38
16	3.31	2.82	2.39
24	5.66	5.61	4.20
36	7.49	6.46	4.02
48	8.82	7.21	4.01

*Phytase activity in original soil in mg. of phytate phosphorus mineralized was 4.30, 2.65, and 0.72 in samples at pH 5, 6, and 7 respectively.

Table 31. Mineralization of phytate phosphorus incubated with different fresh field soils.

Soil	Phytate phosphorus added, mg.	Phytate phosphorus mineralized,* mg.
Carrington silt loam, pH 5.2	4	0.018
	8	0.127
	12	0.315
	16	0.519
	20	0.592
Edina silt loam pH 5.0	4	0.008
	8	0.045
	12	0.115
	16	0.244
	20	0.327
Clarion silt loam pH 7.5	4	0.023
	8	0.092
	12	0.095
	16	0.094
	20	0.079

*Phytase activity in original soil in mg. of phytate phosphorus mineralized was 0.36, 0.18 and 0.12 in the Carrington, Edina, and Clarion soils respectively.

Table 32. Mineralization of phytate phosphorus incubated with limed and unlimed Carrington silt loam.

Phytate phosphorus added, mg.	Phytate phosphorus mineralized at indicated pH values, mg.*		
	pH 4.95	pH 5.4	pH 6.25
4	0.02	0.02	0.01
8	0.06	0.03	0.04
12	0.11	0.09	0.10
16	0.13	0.20	0.16
20	0.31	0.27	0.20

*Phytase activity in original soil in mg. of phytate phosphorus mineralized was 0.20, 0.14, and 0.22 in samples of pH 4.95, 5.4, and 6.25 respectively.

Table 33. Mineralization of phytate phosphorus incubated with Marion silt loam at different pH values with and without added wheat bran phytase.

Phytate phosphorus added, mg.	Phytate phosphorus mineralized at indicated pH values, mg.	
	pH 5	pH 7
	-No phytase added-	
4.0	0.01	-0.01
6.0	0.01	-0.01
7.0	0.15	0.10
8.0	0.19	0.13
8.5	0.14	0.12
9.0	0.18	0.19
9.5	0.31	0.13
10.0	0.21	0.18
10.5	0.35	0.18
11.0	0.32	0.20
11.5	0.36	0.28
12.0	0.57	0.43
13.0	0.46	0.43
15.0	0.62	0.45
18.0	0.72	0.50
22.0	0.69	0.53
	-Phytase added-*	
4.0	-0.01	0.01
6.0	0.16	0.10
7.0	0.39	0.15
9.0	0.90	-
10.0	1.00	0.61
12.0	1.69	1.09
15.0	2.20	1.95
18.0	2.48	2.77
22.0	2.37	3.83

*Phytase activity per ml. of phytase extract with 4 hours' incubation was 3.2 mg. phytate phosphorus mineralized.

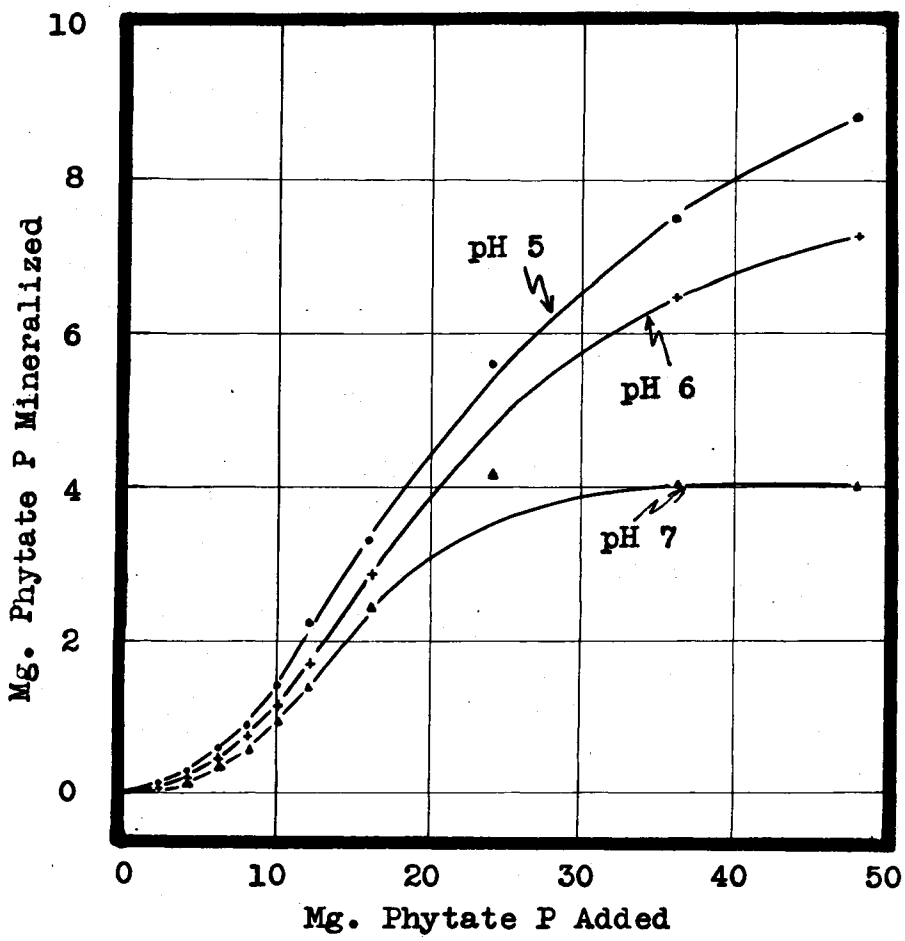

Figure 12. Mineralization of phytate phosphorus incubated with Marion silt loam of pH 5, 6, and 7.

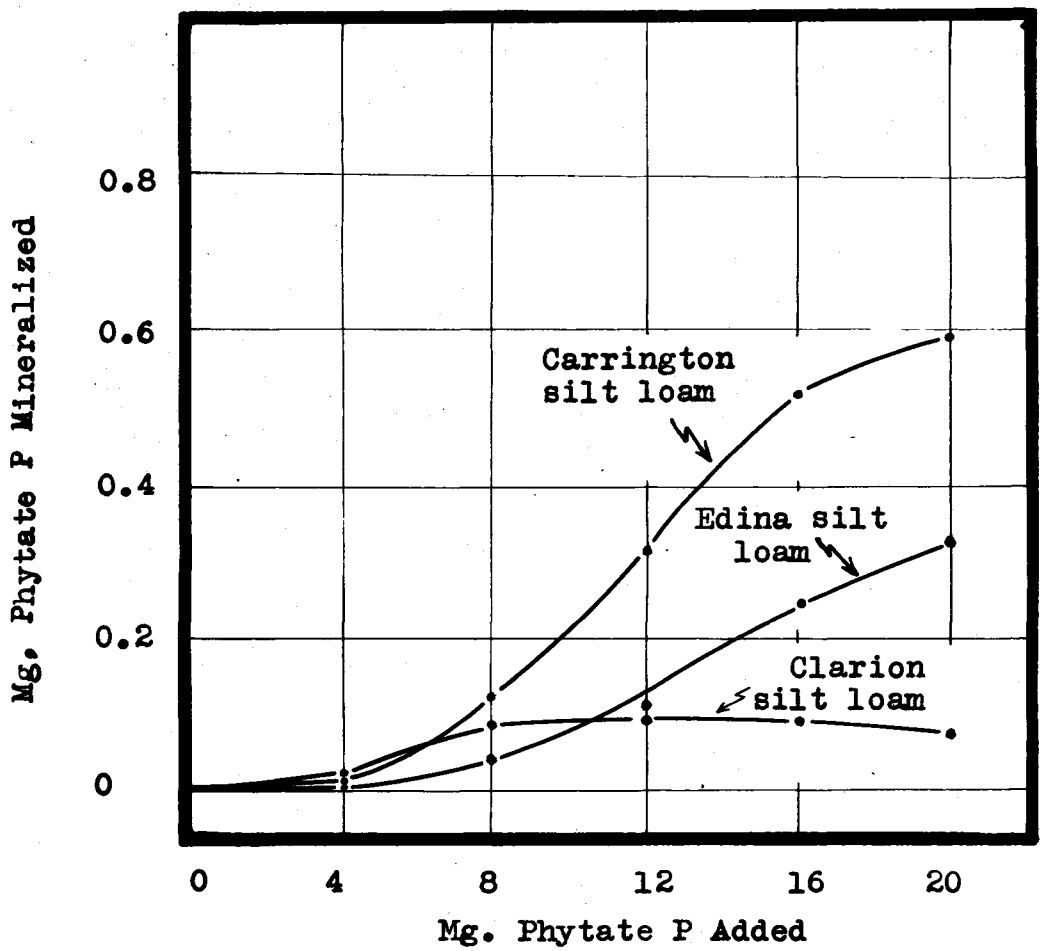

Figure 13. Mineralization of phytate phosphorus incubated with three different fresh field soils.

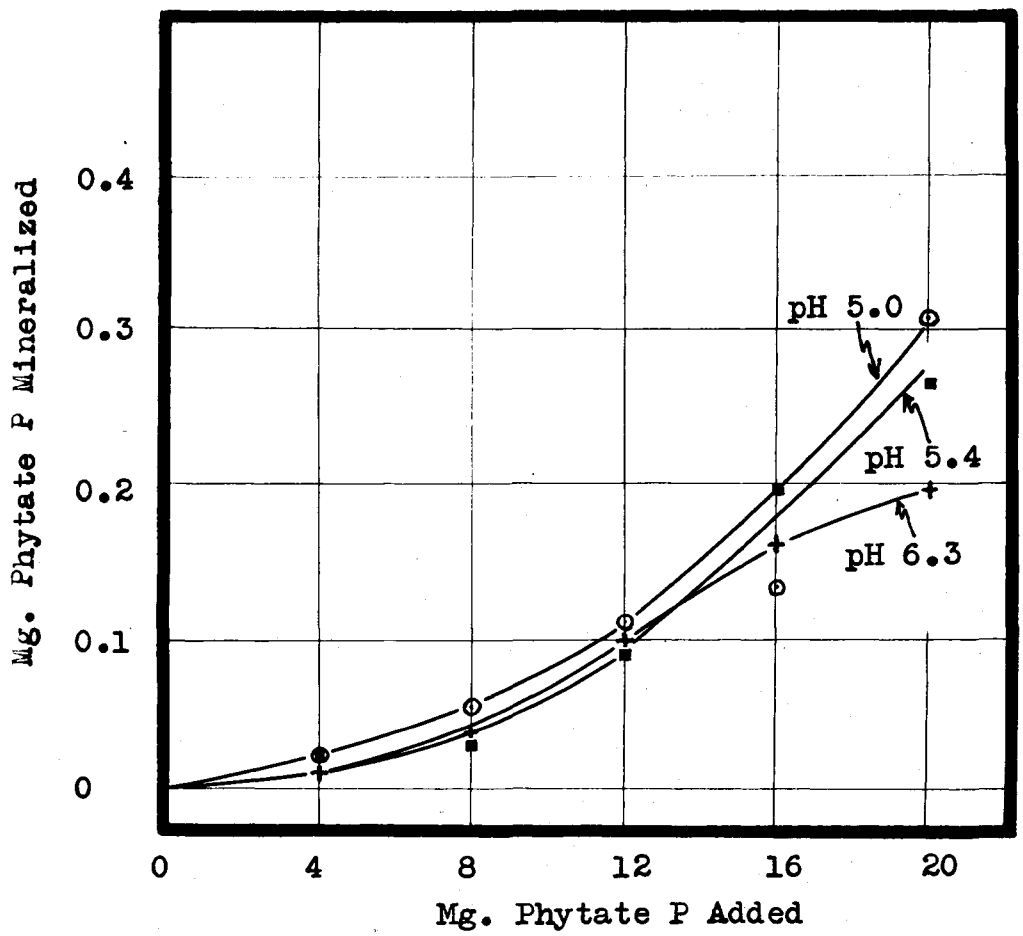

Figure 14. Mineralization of phytate phosphorus incubated with limed and unlimed Carrington silt loam

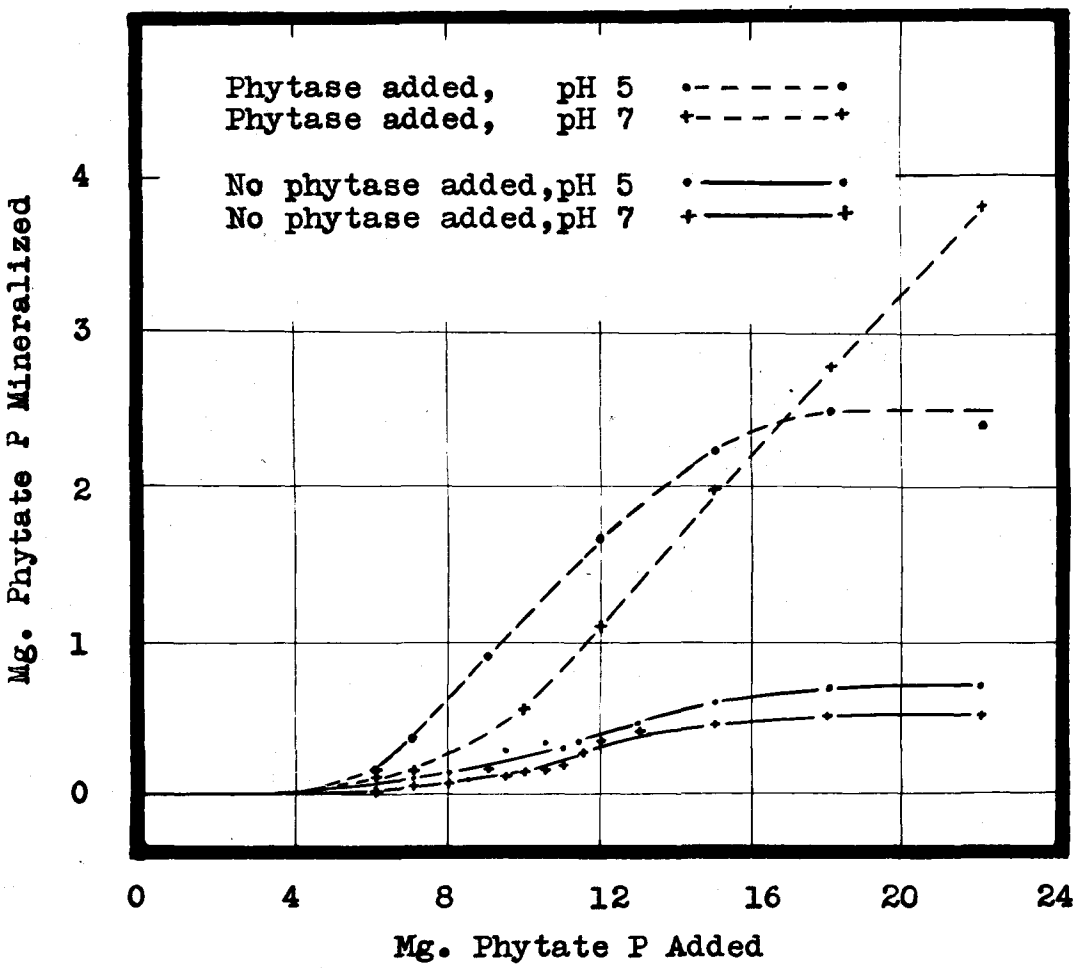

Figure 15. Mineralization of phytate phosphorus incubated with Marion silt loam with and without added wheat bran phytase.

results are confounded with soil type.

An experimental difficulty common to the first three experiments was that the phytase was already in the soil when the phytate was added. The fixation of phosphate and phytate by the soil is not instantaneous and hence for some time during the incubation the phytase was operating in a system where the full solubility differences were not developed. Under such conditions the activity of the enzyme probably plays the major part in determining the rate of mineralization. With the possible exception of the Clarion soil of the second experiment, the results show the general dependence of the mineralization on the effective level of phytase activity.

In the fourth experiment an attempt was made to overcome this defect. The soils had very low initial phytase activities and the storage of the samples after the phytate had been added was designed to allow appreciable fixation to occur. Toluene was added and the storage was in the refrigerator so as to inhibit new microbial development and limit the effect of the phytase already present. The phytase added had an activity, as measured in solution, greatly exceeding that found as yet in any soil. Despite these refinements the experiment did not give the expected results owing to an unforseen factor. It will be noted

that where more than 16 mg. of phytate phosphorus were added the mineralization was greater at pH 7 than at pH 5 despite the presence of much soluble phytate as well as greater phytase activity at pH 5 than at pH 7. This behaviour suggests that the deactivation of the enzyme by the soil was very much greater at the lower pH. This explanation can account for the relative positions of the two curves over most of their lengths. With small amounts of phytate added, the phytase was not working at full capacity in either case and the rate of mineralization was primarily determined by the pH effect on the phytase. As the amount of phytate increased, the enzyme capacity was approached and was reached first in the pH 5 series due to the greater deactivation of the enzyme by the soil. With the pH 7 series this enzyme capacity was not reached because of the greater amounts of active phytase remaining and thus with the larger amounts of the phytate added the mineralization was greater at pH 7 than at pH 5.

It is seen that the pH may affect the solubility of the phytate added to soil, the degree of inactivation of phytase by the soil, and the activity of the remaining phytase. Such a complex system is very difficult to control and it is considered that the approach to the problem used in the present section is not suitable by reason of this complexity.

It is evident from the data reported in this section that the amount of phytase naturally present in soil may be sufficient to mineralize a relatively large amount of phytate phosphorus when the concentration of soluble phytate phosphorus is high. However, in the experiments where the large amount of mineralization occurred the quantity of phytate phosphorus present in the system was far in excess of that naturally present in soils. Had a comparable percentage mineralization of soil phytate phosphorus been accomplished in the same interval of time the amount of inorganic phosphorus produced would be too small to permit accurate measurement. In a series of experiments conducted parallel to those reported in this section but differing in that soil was incubated with increasing amounts of phytase, there was no measurable increase in phosphorus mineralization from added phytase. It is thus still uncertain whether the solubility of phytate or the level of enzyme in the soil is the deciding factor in the stabilization of phytates in the soil.

Determination of Phytate Phosphorus in Soil

The previous mineralization experiments all involved the addition of a soluble phytate to the soil and the determination of the effect of incubation on the added

phosphorus. This method involves working with a system different from that normally found in soil, where the soil phytates may be protected from enzymatic attack. The study of the mineralization of the natural soil phytin *in situ* should thus approach more closely to the natural conditions.

The method of following phytate mineralization by measuring the increase in inorganic phosphorus has the inherent weakness that it does not take into account the possible resynthesis of the mineralized phosphorus into new organic compounds. For this reason it was considered desirable to measure the phytin phosphorus in the soil directly. The methods that were hitherto available were all of a tentative nature and had not been critically evaluated. Accordingly, the development of a new method was undertaken.

Development of method.

A review of the literature showed that the following findings might be used as bases for the method. The first was that of Wrenshall and Dyer (72), who found that soil organic matter could be destroyed by sodium hypobromite oxidation under conditions which did not destroy phytin. Bower (11) confirmed this observation and also found that under these conditions yeast nucleic acid was appreciably

destroyed. Bower's phytate and nucleic acid fractions of the soil organic phosphorus were stable to about the same extent as were their authentic counterparts. The second finding was that of Bower (11), who separated phytin from yeast nucleic acid by the precipitation of the former as the calcium salt under alkaline conditions. He applied this method to soil organic phosphorus and got an apparently effective separation. The third point of interest was the method of Heubner and Stadler (31) for the determination of phytin. They were the first to use ferric chloride in a relatively strong acid medium as a specific precipitant for phytin.

No extensive investigations of methods of extraction from the soil were undertaken. Wrenshall and Dyer (71) found that if the preliminary acid washing was done with hydrochloric acid up to 4 N strength, there was no increase in mineralization of the organic phosphorus due to the increase in acid strength. Bower (10) digested his soils with 0.5 N sodium hydroxide for 4 hours at 85 to 90° C. These workers all found that the amount of organic phosphorus extracted by the preliminary acid washing was small and seldom exceeded 10 per cent of the total organic phosphorus.

The method selected was to wash the soil with 1 N hydrochloric acid until the filtrate was free of calcium

and then to digest the soil with 0.5 N sodium hydroxide for 3 hours at 80 to 90° C, using a soil to sodium hydroxide solution ratio of 1:10. Only the sodium hydroxide extract was used in the developmental work.

The essential problem was to separate the phytates from the other organic compounds which contain phosphorus and which are classified as nucleic acids, and this required the removal of the soil organic matter with no loss of phytates in the process. Hypobromite oxidation appeared to offer a means of accomplishing this objective, especially if the stability of the phytates were increased by making them insoluble. This scheme was tested as described below.

Known amounts of sodium phytate, sodium phytate derivatives and yeast nucleic acid were added to 10 ml. samples of a sodium hydroxide extract of soil. One hundred ml. of a clear, saturated solution of calcium hydroxide were added to precipitate the soil phytates and the added phytates and phytate derivatives. Forty ml. of bromine water were then added and the samples were digested for 8 hours at 80 to 90° C. At the end of this time the mixtures were a clear golden color and contained voluminous white precipitates. These precipitates were filtered off, washed with calcium hydroxide solution and analyzed for organic phosphorus. Checks were run with no organic phos-

phorus added. The recoveries of the added organic phosphorus were 50 per cent for sodium phytate, 24 per cent for the phytate derivatives, and 14 per cent for the yeast nucleic acid. It was concluded from these results that a method based entirely on preferential oxidation of the nucleic acid would not be likely to be successful and accordingly the idea was dropped.

Experiments were then conducted to see if the soil organic matter could be removed by hypobromite oxidation under conditions such that the phytates would not be destroyed. Liquid bromine was added to the samples of the sodium hydroxide extract with and without calcium hydroxide added. Various ways of heating the mixtures were tried and the results were checked by determining the recovery of added phytate phosphorus. Consistent results could not be obtained. The variation seemed to depend on the degree of oxidation of the organic matter, which in turn depended on the method used. If the sodium hydroxide was heated to boiling before the addition of bromine, the color of the solution obtained on subsequent boiling was lighter than it was if all the heating took place after the bromine was added. This behavior suggests that two types of reactions were taking place: oxidation favored by high temperatures, and stabilizing brominations favored

by low temperatures. In all cases the final solutions contained a white, gelatinous precipitate. An analysis of a thoroughly washed sample of this precipitate showed that it did not contain organic phosphorus, confirming Bower's (10) findings. However this precipitate was variable in quantity, tending to be smaller when the oxidation was greater, and there was a general correlation between the recovery of the added phytate phosphorus and the size of the precipitate. It was concluded that the erratic results were due to the variable amounts of this gelatinous precipitate containing adsorbed organic phosphorus. Overcoming this difficulty by thorough washing of the precipitate was considered to be impractical, since the washing partially dissolved the precipitate. The use of bromine was dropped and the possibilities of acid precipitation of the organic matter were investigated.

Bower (10), in agreement with Yoshida (73), had found that if the reaction of the sodium hydroxide extract was adjusted to pH 2.5, a precipitate of organic matter was obtained and over 95 per cent of the organic phosphorus remained in the filtrate. Stanford (64), however, was not able to confirm this behavior and found that the amount of organic phosphorus remaining in the filtrate was relatively low and variable between soils. He tested the effect of pH of organic matter precipitation using an

extract from one soil and found that in the range from pH 1 to pH 2 there was no appreciable change in the percentage of the total organic phosphorus passing into the filtrate.

To see whether the acid-precipitation could be used to advantage in the present work, the effect of pH was investigated over a wider range than that employed by Stanford (64). Twenty-five ml. of an 0.5 N sodium hydroxide extract of Muscatine silt loam were pipetted into two series of beakers. To one series a known amount of sodium phytate was added. The pH values of both series were adjusted over the range from pH 0.1 to pH 2.5 with hydrochloric acid. The samples were then warmed to flocculate the organic matter, which was centrifuged off and washed with 10 ml. of hot 0.05 N hydrochloric acid. The washings were added to the filtrate, which was then made to volume and analyzed for organic phosphorus. The results are given in Tables 34 and 35 and in Figure 16.

These results show that as the pH was lowered there was a consistent and progressive increase in the amount of phosphorus that remained in the filtrate. This amount was at a near possible maximum at pH 0.1. To see if similar high recoveries in the filtrate could be obtained in the case of other soils, the sodium hydroxide extracts of several soils were similarly treated at pH 0.1. It was found that the percentages of the total organic phosphorus

Table 34. Soil organic phosphorus in the filtrate after the removal of organic matter in an alkali extract of Muscatine silt loam by acid precipitation at different pH values.

pH	Organic phosphorus in the filtrate	
	Milligrams*	Per cent of total
0.1	0.343	93.3
0.2	0.339	92.1
0.3	0.334	90.8
0.85	0.263	71.5
1.20	0.208	56.5
1.78	0.127	34.5
2.28	0.103	28.0
2.48	0.095	25.8

*Total soil organic phosphorus, 0.368 mg.

Table 35. Added phytate phosphorus in the filtrate after removal of organic matter in an alkali extract of Muscatine silt loam by acid precipitation at different pH values.

pH	Organic phosphorus in filtrate, mg.	Recovery of added phytate phosphorus*	
		Milligrams	Per cent
0.10	1.027	0.684	105
0.70	0.904	0.614	94.5
1.23	0.564	0.362	55.6
1.40	0.414	0.239	36.8
1.65	0.292	0.145	22.3
2.48	0.136	0.041	6.3
3.40	0.114	-	-

*Phytate phosphorus added, 0.650 mg. Recovery found by graphical subtraction using interpolated values from Figure 16.

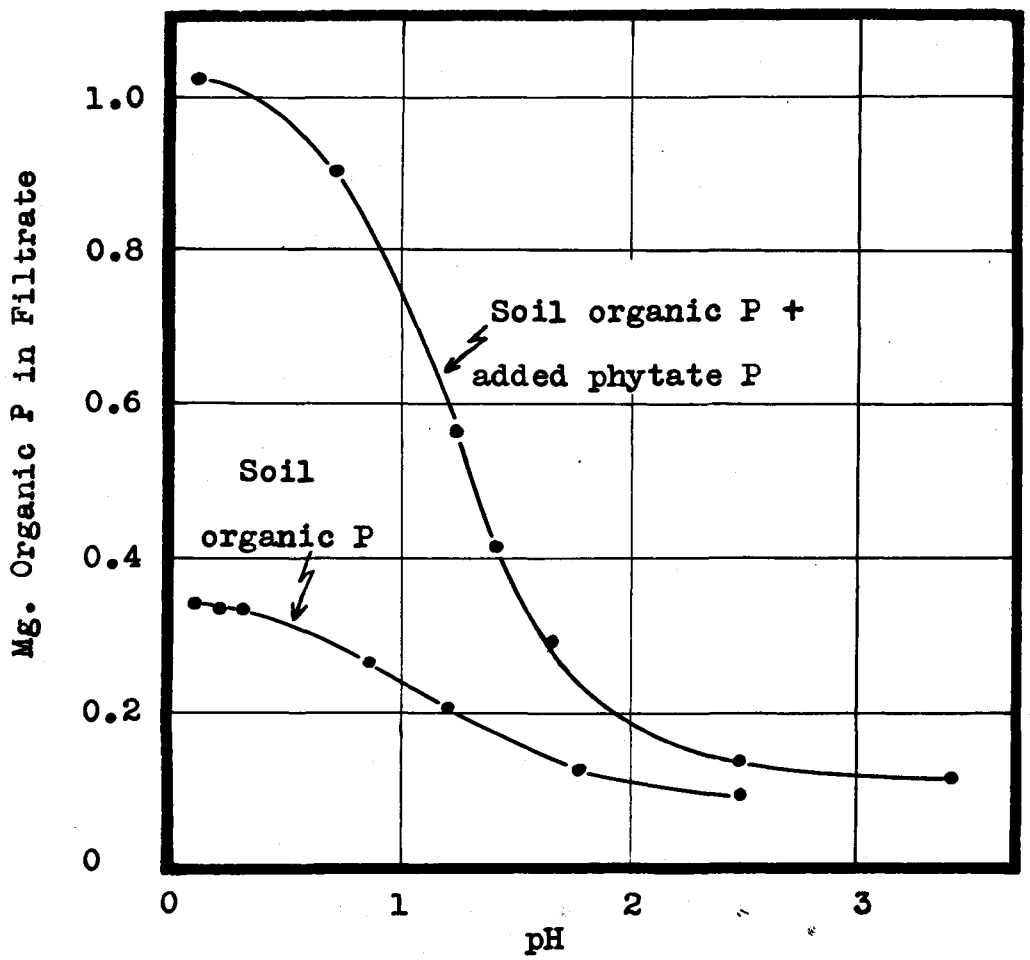

Figure 16. Soil organic phosphorus and added phytate phosphorus in the filtrate after removal of organic matter in an alkali extract of Muscatine silt loam by acid precipitation at different pH values.

remaining in the filtrate were 95, 88, 91 and 94 for samples of Carrington silt loam, Fayette silt loam, Marshall silt loam, and Tama silt loam, respectively. These results are comparable to that found with the samples of Muscatine silt loam.

The fact that the recovery of the added phytate phosphorus was complete at pH 0.1 suggests that the soil organic phosphorus remaining with the precipitated organic matter was not of phytin form. This point was checked by dissolving a known amount of the precipitated organic matter in 50 ml. of 0.5 N sodium hydroxide, adding 0.5 ml. of liquid bromine, and heating the mixture on the steam chest. At the same time another sample of the same organic matter to which a known amount of sodium phytate had been added was treated in the same manner. At the end of 2 hours' heating calcium hydroxide was added and the resulting precipitate was centrifuged down, washed with calcium hydroxide and analyzed for organic phosphorus. It was found that only 4 per cent of the soil organic phosphorus was recovered whereas the recovery of the added phytate phosphorus was 86 per cent. It was concluded that such a wide difference between the recoveries of the soil organic phosphorus and of the phytate phosphorus indicated that if there was any phytin in the organic matter precipitate it was a negligible amount. The removal

of most of the organic matter by acid precipitation at a pH of 0.1 was therefore considered to be a satisfactory method for the purpose of separating phytate phosphorus from the bulk of the organic matter.

These results contradict the findings of both Bower and Stanford recorded above, but no explanation of the contradiction can be given. A possible reason for the change in the amount of phytate phosphorus that remained in the filtrate as the pH was changed is provided by the work of Fontaine et al. (23) on the extraction of organic nitrogen and phytin from soybean and peanut meals. They found that the ease of extraction of these materials depended on the pH of the system. The pH of minimum extraction of the phytin was between 3 and 4 while for nitrogen it was rather higher. As the pH was changed in either direction from this range the amount of both nitrogen and phytin extracted increased considerably. This behavior was explained by postulating the existence of a phytin-protein complex of maximum stability over the pH range approximately 3 to 4. A similar phytate-soil proteinaceous material complex may be the reason why the amount of added phytate phosphorus that remained in the filtrate varied with the pH. The solubility curve for the added phytate shown in Figure 16 is very similar to that given by Fontaine et al. (23).

Calculations of the amount of phytate phosphorus that could be expected in the filtrate after the organic matter had been removed, showed that with a soil to sodium hydroxide solution ratio of 1 to 10, about 50 ml. or more of the extract would be required. It was desired to reduce this volume to facilitate handling and the best method to do this appeared to be the precipitation of the phytate phosphorus. An iron precipitation under acid conditions was considered preferable to the calcium precipitation in an alkaline medium, since the solubility studies had demonstrated the great insolubility of both ferric phytate and ferric phytate derivatives. Furthermore, it was found that when the filtrate from the organic matter separation was made alkaline, most of the remaining organic matter was precipitated.

Earley (19) precipitated phytin with ferric chloride in a medium containing 4 per cent of sodium sulfate and 0.6 per cent of free hydrochloric acid. A preliminary test showed that the pH of this system was 1.7 to 1.8. His method, slightly modified, was adopted and all subsequent iron precipitations were carried out at pH 1.7 to 1.8 as determined with a glass electrode. As an initial check of the method determinations of known amounts of sodium phytate and sodium phytate derivatives were made. The known solutions were pipetted into 50-ml. centrifuge

tubes followed by 2.5 ml. of a solution of ferric chloride containing about 1.3 mg. of iron per ml. Then 12 ml. of a 10 per cent solution of sodium sulfate were added and the pH was adjusted with dilute hydrochloric acid. The volume was made to 30 ml. in all cases. The precipitate which appeared was flocculated by heating the tube in the water bath for 15 minutes. The precipitate was then centrifuged down and the supernatant liquid was decanted off. A little dilute sodium hydroxide was added, whereupon the precipitate readily dissolved, giving a clear, brown-colored solution. The solution was made to volume and the organic phosphorus was determined. The results are given in Table 36.

Table 36. The determination of phytate and phytate derivative phosphorus by iron precipitation.

Material	Organic phosphorus added, mg.	Organic phosphorus measured	
		Milligrams	Per cent of added
Sodium phytate	0.650	0.636	98
	0.650	0.655	101
Sodium phytate derivatives	0.602	0.603	100
	0.602	0.605	100

The results show that the precipitation was quantitative under the conditions used. Accordingly, the same pro-

cedure was used with the filtrate of the sodium hydroxide soil extract from which the major portion of the organic matter had been removed by acid precipitation at pH 0.1. Fifty-ml. samples of the sodium hydroxide extract were used and known amounts of sodium phytate were added to one series. The final volume after the reagents and washings had been added was about 80 ml., and accordingly 3.2 g. of solid sodium sulfate were used. On placing the tubes in the water bath a brown flocculant precipitate quickly appeared where the phytate had been added but with the extract alone this precipitate only appeared slowly. The centrifuged precipitate was dissolved in a few ml. of 1:1 ammonium hydroxide in preference to sodium hydroxide since the latter contained a little inorganic phosphorus. The results are given in Table 37.

The results show that there was full recovery of the phytate phosphorus and that only about 50 per cent of the soil organic phosphorus was precipitated. However the precipitate contained extraneous organic matter. The removal of the excess organic matter was accomplished by sodium hypobromite oxidation. The precipitates were dissolved in 15 ml. of approximately 1 N sodium hydroxide and 0.2 ml. of liquid bromine was added. The tubes were then placed in the water bath and heated to the boiling point until the mixtures were a clear, golden color and

Table 37. The iron precipitation of soil organic phosphorus and added phytate phosphorus from a soil extract.

System	Phytate phosphorus added, mg.	Organic phosphorus measured, mg.	Phytate phosphorus recovered	
			Milligrams	Per cent
Extract alone	-	0.330*	-	-
	-	0.323	-	-
Extract plus phytate	0.650	1.031	0.704**	108
	0.650	1.002	0.675	104

*The total organic P in the original extract was about 0.6 mg.

**By subtraction using the average figure for the extract alone

the ferric hydroxide produced was well flocculated. After the tubes were cooled concentrated hydrochloric was added until the color of free bromine appeared. This color was then discharged by adding a solution of sodium sulfite. The resulting solution was colorless and clear. To see if there had been any decomposition of the added phytate by this treatment the organic phosphorus was again determined by iron precipitation. The results are given in Table 38.

Table 38. The iron precipitation of soil organic phosphorus and added phytate phosphorus after sodium hypobromite oxidation of the organic matter.

System	Phytate phosphorus added, mg.	Organic phosphorus measured, mg.	Phytate phosphorus recovered	
			Milligrams	Per cent
Extract alone	-	0.135	-	-
	-	0.104	-	-
Extract plus phytate	0.650	0.809	0.689	106
	0.650	0.805	0.685	105

The recovery of the added phytate phosphorus was complete. The soil organic phosphorus was still further reduced by the treatment but no check was made to determine whether the bromine treatment or the additional iron precipitation was responsible.

In the treatments used thus far there had been no

definite attempt to separate the phytin from other organic compounds containing phosphorus. Accordingly, a calcium precipitation was included at this stage. It had been noted in the previous experiments that the recovery of the added phytate phosphorus always exceeded the theoretical. The reason was not apparent, but it was thought possible that the ferric hydroxide might be carrying with it adsorbed organic phosphorus. For this reason the calcium precipitation experiment was divided into two parts; in one the ferric hydroxide was removed after the bromine treatment, while in the other it was allowed to remain during the calcium precipitation.

Known amounts of sodium phytate and yeast nucleic acid were added to 50-ml. samples of the sodium hydroxide extract. The organic matter was then removed, the organic phosphates were precipitated with iron and the organic matter of these iron precipitates was treated with bromine, as described above. At this stage the ferric hydroxide of one series was removed by centrifugation, the precipitate was washed with 5 ml. of hot 0.5 N sodium hydroxide and the washings were returned to the centrifugate. All the samples were thereafter treated alike. After the samples had been made acid and the color of free bromine had been discharged with sodium sulfite, they were made alkaline to phenolphthalein with sodium hydroxide. Ten ml. of

clear, saturated calcium hydroxide solution were added and the tubes were then placed in the water bath to flocculate the resulting precipitate. The precipitate was centrifuged down and washed with 10 ml. of the calcium hydroxide solution.

In a previous experiment carried up to this stage and with the ferric hydroxide present, analysis of the calcium precipitate had shown that the recovery of the added phytate phosphorus was about 106 per cent and that of the nucleic acid was from 10 to 23 per cent. The calcium precipitation had not been effective. In the present experiment, therefore, the calcium precipitate was dissolved in 0.5 ml. of concentrated hydrochloric acid and the iron precipitation was repeated. The organic phosphorus in the iron precipitate was then determined. The results are given in Table 39.

It is seen from these data that when the ferric hydroxide was removed the recoveries were close to the theoretical. They were still slightly high but it was considered that the additional work necessary to correct this error was not warranted.

In addition to the above preliminary work studies were made of the variation in precipitation when different amounts of ferric chloride and calcium hydroxide were used. In neither case was the quantity critical. Ferric chloride

Table 39. The recovery of phytate and nucleic acid phosphorus by combined iron and calcium precipitations.

System	Organic phosphorus added, mg.	Organic phosphorus measured, mg.	Added organic phosphorus recovered	
			Milligrams	Per cent
	-Ferric hydroxide present-			
Extract alone	-	0.159	-	-
	-	0.161	-	-
Extract plus phytate	0.325	0.520	0.360	111
Extract plus nucleic acid	0.156	0.179	0.019	14
	0.156	0.171	0.011	7
	-Ferric hydroxide absent-			
Extract alone	-	0.144	-	-
	-	0.148	-	-
Extract plus phytate	0.325	0.481	0.335	103
Extract plus nucleic acid	0.156	0.149	0.003	2
	0.156	0.150	0.004	3

should not be used in excesses much greater than about 5 times the equivalent of phytate present. At greater excesses there was full precipitation on heating the mixture but the formation of the precipitate was rather slow. The amounts given in the following detailed account of the method were found to give good results.

Method.

Fifteen g. of the air-dried soil were weighed into a beaker. Fifty ml. of 1 N hydrochloric acid were added and the mixture was stirred and allowed to stand for 30 minutes. It was then filtered and the soil was washed with 200 ml. of 1 N hydrochloric acid followed by 4 washings with water. The washed soil and the paper were then added to 150 ml. of 0.5 N sodium hydroxide in a 250 ml. beaker. The paper was broken up with a stirring rod and the beaker was covered with a watch glass and placed on the steam chest at 80 to 90° C for 3 hours. At the end of this time the sample was filtered on a Buchner funnel and thoroughly washed with water. The filtrate was cooled and diluted to 250 ml.

Fifty ml. of the sodium hydroxide extract were pipetted into a 250 ml. beaker and concentrated hydrochloric acid was added until the pH was 0.1 as determined with a

glass electrode. About 7 ml. of acid were usually required. The mixture was warmed on the steam chest to flocculate the precipitated organic matter and then quantitatively transferred to a suitable centrifuge tube, washing the beaker with a little water. The organic matter was centrifuged down and the centrifugate was poured into a 250 ml. beaker. The precipitate of organic matter in the centrifuge tube was suspended in 10 ml. of hot 1.0 N hydrochloric acid added rapidly with a pipette and was again centrifuged down. The washings were added to the original centrifugate.

To this acid solution were added 3.2 g. of anhydrous sodium sulfate or sufficient to give a final concentration of 4 per cent. Two ml. of a solution of ferric chloride* were then added followed by sufficient 30 per cent sodium hydroxide to give a pH of 1.7 to 1.8 as measured by a glass electrode. The beaker was then placed on the steam chest until the brown iron precipitate was well flocculated. If the phytate concentration was high this required about 10 minutes' heating but if it was low the flocculation was aided by vigorous stirring and heating up to half an hour. When the precipitate was well flocculated the mixture was transferred to a centrifuge tube

*The reagent chemical was dissolved in water and stabilized with a little hydrochloric acid. The solution contained about 1.3 mg. of iron per ml.

and the precipitate was centrifuged down. The supernatant liquid was poured off and discarded. Fifteen ml. of 1 N sodium hydroxide were added to the precipitate. The tube was swirled until the precipitate had fully dispersed and was then placed in a warm water bath (about 40° C) and 0.2 ml. of bromine was added. The temperature of the bath was raised to the boiling point and heating was continued until the solution was a clear, golden color and the ferric hydroxide was well flocculated. During the early stages of heating it was necessary to stir the solution to prevent explosive vaporization of the bromine. The ferric hydroxide was then centrifuged down and the supernatant liquid was poured into another 50 ml. centrifuge tube. The precipitate of ferric hydroxide was broken up by rapidly adding 10 ml. of hot 0.5 N sodium hydroxide from a pipette and was again centrifuged down. The washings were added to the centrifugate. Hydrochloric acid was added dropwise until the solution was colored with free bromine and the color was discharged by the dropwise addition of a saturated solution of sodium sulfite.

The water-clear solution was made alkaline to phenolphthalein with 30 per cent sodium hydroxide added drop by drop and then 10 ml. of a saturated solution of calcium hydroxide were added. The precipitate, which appeared immediately, was flocculated by placing the tube

in the hot water bath, after which it was centrifuged down and washed with 10 ml. of the calcium hydroxide solution in the same manner as described for the washing of the ferric hydroxide precipitate above. The centrifugate and washings were discarded.

The calcium precipitate was dissolved with 3 drops of concentrated hydrochloric acid and was then transferred to a 50 ml. beaker with three washings of 5 ml. of water. One g. of anhydrous sodium sulfate was added followed by 0.5 ml. of ferric chloride solution (described previously), after which the pH was adjusted to 1.7 to 1.8 with 1 N sodium hydroxide using the glass electrode. The final volume was about 25 ml. The beaker was placed on the steam chest and when the precipitate had flocculated the contents were transferred to a 50 ml. centrifuge tube and the precipitate was centrifuged down. The supernatant liquid was then gently sucked off using a fine jet attached to the water pump. The previous precipitates adhered to the tube very well but this iron precipitate did not and the removal of the liquid had to be done with care. This precipitate was washed with 10 ml. of 0.5 per cent hydrochloric acid* in the same manner as for the other washings, the washings being sucked off carefully.

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*1 ml. of concentrated acid diluted to 200 ml. with water

A few ml. of 1:1 ammonium hydroxide were added and the precipitate was dispersed by swirling the tube. This clear, pale-brown solution was transferred to a 50 to 100 ml. volumetric flask, depending on the amount of phosphorus present, made to volume and the organic phosphorus was determined by the method of Dickman and Bray (17).

Evaluation of the method.

In the exploratory recovery experiments only yeast nucleic acid, predominantely ribonucleic acid, was compared to phytate. It was possible that other forms of organic phosphorus might not be successfully eliminated and accordingly the method was used with samples of a sodium hydroxide extract of soil containing known amounts of various compounds containing phosphorus. The results are given in Table 40.

None of these forms of organic phosphorus have been found in soil but they are representative of phosphated compounds important in the metabolic processes of living organisms and together with ribonucleic acid constitute the main portion of the organic phosphorus in plants and micro-organisms with the exception of the phytate phosphorus in seeds. The method successfully eliminates them all; hence, it can be considered to be reasonably specific for inositol bound phosphorus.

Table 40. The recovery of various forms of organic phosphorus added to a sodium hydroxide extract of soil when analyzed for phytin phosphorus.

Compound added	Organic phosphorus added, mg.	Organic phosphorus measured, mg.	Added organic phosphorus* recovered, Milligrams	Added organic phosphorus* recovered, Per cent
Fructose 6-phosphate	0.560	0.370	-0.006	-1.7
Fructose 1,6-diphosphate	0.670	0.377	0.001	0.1
Glucose 1-phosphate	0.490	0.378	0.002	0.4
Adenosine triphosphate	0.445	0.381	0.005	1.1
Adenylic acid	0.620	0.378	0.002	0.3
Desoxyribonucleic acid	0.805	0.372	-0.004	-0.5

*Soil organic (phytate) phosphorus, 0.376 mg.

The method is considered to be basically sound but is still subject to possible errors concerning which further work is required. The points which require clarification or simplification are as follows.

It is not known whether or not the acid soil extract should be included in the determination. As yet there is insufficient evidence upon which to assess this factor although it is not considered that the acid washing removes much, if any, phytin. This point was checked in only one instance using a Carrington soil which had been limed to pH 6.25 and which had a very high phytin phosphorus content. With the acid extract included the amount of phytin phosphorus in the soil was 139.9 p.p.m. while if only the sodium hydroxide extract was used the value was 141.3 p.p.m.

Through an oversight the effect of the hypobromite oxidation and the subsequent calcium precipitation on the recovery of added phytate derivatives was not studied. In previous trials where calcium was added prior to the hypobromite oxidation the recovery of the added phytate phosphorus was complete but that of the phytate derivatives was 80 to 90 per cent. It is probable that some loss of phytate derivatives occurs at this stage. Since this form probably constitutes about one third of the total inositol-bound phosphorus the resultant error is

not large.

The final iron precipitate is not pure. If the iron of this precipitate was removed and the calcium salt was formed and then dissolved in 2 to 3 N hydrochloric acid and heated to 140° C the solution became pale yellow in color and specks of black carbon appeared. A solution of authentic sodium phytate similarly treated remained colorless and clear. It appears unlikely that this contaminating material contained organic phosphorus and it is suggested that it might have been a small amount of the gelatinous material produced by the hypobromite oxidation.

The reproducibility of the method is variable. Within any one series of determinations run at the same time the precision was generally found to be good. If however a new series was run later, the new results agreed well within themselves but differed from the previous ones by a relatively constant amount. The average maximum difference was 15 per cent. However, since only differences in phytin content were required in the present work the method was considered to be adequate for the purpose.

The method is long and laborious. This is partially due to the necessity of adjusting the pH for the iron precipitations with the glass electrode. Quinaldine red and 2,4-dinitro phenol were tried as indicators but it was not

found possible to consistently reach a value of pH 1.8 when they were used. There may be a pH range downward from 1.8 over which the iron precipitation is quantitative, since good precipitation has been noted at pH values as low as 0.85. If this is so, then indicators may well replace the glass electrode.

Mineralization of Soil Phytate Phosphorus

In the previous mineralization studies, attempts were made to determine if the insolubility of the phytates in soil is the factor that limits the mineralization of phytate phosphorus. In the present work the objective was the same but the natural phytin phosphorus in the soil was utilized.

Method.

Six soils high in organic phosphorus were used. Each soil was air dried, passed through a 2 mm. sieve and adjusted to pH values of 5, 6 and 7 as described on page 108.

Three samples of 100 grams of each soil at each pH were weighed into mason jars. All were placed in incubation cabinets for 15 weeks at room temperature. During this time each pH series received the following treatments. One series was moistened with water, another received

water and also energy material and nitrogen in solution form, while the third was left dry as a check. Each was replicated twice.

The additions were made for the most part at biweekly intervals. The moisture content was kept at 25 to 35 per cent of the dry soil weight but occasionally the soils were allowed to dry out fully so as to stop anaerobic conditions from developing and also to initiate new bursts of microbial activity. For the first month the energy material was soluble starch but later this was replaced by sucrose. The nitrogen was in the form of ammonium nitrate for the first ten weeks but calcium nitrate was substituted for this from then on. The starch and sugar were added at the rate of 0.5 per cent of the soil weight and the nitrogen at 0.05 per cent of the soil weight.

At varying times during the incubation the pH values of the series receiving additions were checked. It was found that they were decreasing in all cases, especially in the pH 7 series receiving the energy material and nitrogen. Where necessary additional calcium hydroxide was added to return the reactions to the original values. At the end of incubation the samples were air dried and the replicates were combined. They were then ground, passed through a 40-mesh sieve and the phytate phosphorus was determined.

Results and discussion.

The results are given in Table 41. In Table 42 are

Table 41. The phytate phosphorus content of 6 soils after 15 weeks' incubation dry, moist, and moist with amendments added.

Soil	pH of soil		Phytate P in soil after incubation under indicated conditions, p.p.m.		
	Natural	During incubation	Dry	Moist	Moist plus amendments
Carrington silt loam (6 analyses)	4.9	5	124.0	128.9	129.9
		6	130.2	132.5	122.4
		7	121.0	124.7	112.3
Muscatine* silt loam (2 analyses)	5.6	5	84.5	83.4	84.1
		6	84.6	80.5	78.7
		7	79.4	78.0	67.1
Fayette** silt loam (2 analyses)	5.6	5	83.5	80.4	79.8
		6	80.3	80.6	72.9
		7	79.7	79.5	66.2
Tama silt loam (4 analyses)	5.5	5	74.3	74.0	70.4
		6	73.5	70.0	60.2
		7	70.4	71.5	52.2
Marshall silt loam (1 analysis)	5.3	5	65.8	66.7	62.2
		6	67.8	68.6	54.5
		7	64.4	63.2	52.8
Webster* silty clay loam (5 analyses)	6.0	5	34.7	34.0	32.5
		6	37.3	33.9	31.3
		7	31.8	31.2	27.0

*Calculated on basis of air-dry soil; others calculated on basis of oven-dry soil.
**Virgin soil; other soils cultivated.

Table 42. The decrease in phytate phosphorus content of soils incubated moist and moist with amendments added.

Incubation pH	Decrease in phytate phosphorus with indicated incubation treatments, p.p.m.	
	Water alone	Water amendments
5	0.1	- 1.3
6	-1.3	- 9.0
7	0.2	-11.5
Mean	-0.3	- 7.3

given the mean differences between the phytate phosphorus concentrations of the treated and check samples. The incubation pH values are classification values rather than accurate values since the reactions varied during incubation.

These differences indicate that with only water added there was essentially no mineralization of the soil phytate phosphorus at any pH value. Although the data were not analyzed statistically it is considered that the decrease of 1.3 p.p.m. at pH 5 and with amendments added is not significant but that the decreases at pH 6 and pH 7 are significant decreases.

The lack of mineralization at pH 5 may be accounted for on the basis that the phytates were essentially insoluble at this pH value and hence were not mineralized to a measureable degree under the influence of a high level of phytase activity. On the other hand the definite min-

eralization at pH 6 and 7 may be accounted for on the basis that both the level of phytase activity and the solubility of the phytates were limiting factors. The situation as regards phytate mineralization found in the laboratory may be found in the field also. The phytate phosphorus analyses on different soils given in Table 43 show that the values tended to be lower as the soils became less acid. Hence the apparent decrease in phytate phosphorus may result almost entirely from the greater solubility of the phytates at the higher pH values. It would be expected, however, that in calcareous soils a high content of phytate phosphorus might be found owing to the insolubility of the phytates and the low phytase content and activity found therein.

Although the explanation advanced in the previous paragraph will account for the experimental results it is by no means certain that the situation is as simple as indicated. The tacit assumption is made that during the incubation there was no synthesis of phytates by microorganisms. Analyses of two samples of soil showed that such synthesis may occur. Subsoil samples of Nacogdoches clay of pH 4.6 and Clarion silt loam of pH 8.3, and containing much free calcium carbonate, had been incubated with sugar and nitrogen for 10 weeks. Initially there was no organic phosphorus in these soil materials but at the

Table 43. The phytate phosphorus content and pH of various soils.

Soil number	Soil type	pH	Phytate phosphorus, p.p.m.
3201	Carrington silt loam	4.9	124
3200	Muscatine silt loam	5.6	85
3199	Fayette silt loam	5.6	84
3202	Tama silt loam	5.5	74
3205	Marshall silt loam	5.3	66
3203	Webster silty clay loam	6.0	35
3206	Clarion silt loam	7.5	30
3197A	Carrington silt loam*	4.95	149
3197B	Carrington silt loam	5.45	149
3197C	Carrington silt loam	6.25	141

*Soils from nearby plots which had received 0, 2½, and 10½ tons of lime respectively.

end of the incubation, analysis showed that they contained about 120 p.p.m. of organic phosphorus. These incubated soils were analyzed for phytate phosphorus. None was found in the acid soil but there was a considerable amount in the Clarion soil. When the extracts of both soils was made acid after the bromination treatment, an opalescent precipitate appeared which could be extracted with ether. Since fatty material was probably present it was possible that phospholipids might also be present in abnormally large amounts. Accordingly, the Clarion soil was continuously extracted with absolute alcohol for 4 hours followed by 3 hours with ether. This treatment did not remove all the fatty material, and so after the organic matter had been removed from the sodium hydroxide extract, the acid filtrate was further extracted with ether. The results were 35 p.p.m. and 36.5 p.p.m. of phytate phosphorus with and without solvent extraction, respectively. It was concluded that if the method of determination is as specific as it appears to be, there must have been some phytate synthesis by microorganisms.

On the basis of relative solubilities, a greater accumulation of phytate phosphorus would have been expected in the acid soil than in the calcareous soil. That this

did not occur may have been due to the high phytase activity in the acid soil which mineralized the phytate phosphorus before it reacted with the soil. It can be inferred from the previous work on phytase that in the Clarion soil both the rate of production of the phytase and the effect of pH on the phytase would produce a much lower activity. The result may have been that the rate of phytate synthesis was greater than the rate of mineralization and accumulation occurred. This situation would be favored by the high calcium content of the soil which would tend to stabilize the phytates once formed. A further possibility is that with the wide difference in pH the types of microbial population in the two soils might have been very different.

If this evidence for the synthesis of phytates by soil micro-organisms is substantiated, then the phytate phosphorus status of a soil at any one time would be the result of the synthesis and destruction of the phytates. Any increase or decrease would depend on the relative rates of these two processes. While synthesis may account for the presence of phytates in soil, the results of the mineralization experiment and the analyses of various soils given in Table 43, indicate that the relative solubility of the phytates is the factor determining the amount and stability of phytate phosphorus in different soils.

SUMMARY

Previous investigations indicate that inositol phosphates comprise a large portion of the organic phosphorus in soils. The present investigation was conducted to obtain information on the behavior of inositol phosphates that would contribute to an understanding of the importance of these phosphates in plant nutrition.

Laboratory studies were carried out on the solubility and phytase-catalyzed hydrolysis of the iron, aluminum, calcium and magnesium salts of phytic acid and phytic acid derivatives. This work was followed by studies on the source of phytase in soil, the relationship between the phytase activity and the overall microbiological activity in soils and the effect of the variation of some soil properties on the level of phytase activity. Finally, experiments were conducted to determine the relative importance of phytate solubility and phytase activity in controlling the rate of mineralization of soil phytates. In the course of this work methods were developed for preparing relatively pure sodium phytate, for measuring the phytase activity in soils and for determining the

phytate phosphorus content of soils.

A solution of sodium phytate was prepared from commercial calcium phytate. The original, impure, calcium salt was dissolved in 2 per cent hydrochloric acid and the extraneous matter was filtered off. The calcium salt was reprecipitated with sodium hydroxide, filtered and then redissolved in 8 per cent acetic acid, from which it was reprecipitated in relatively pure form by boiling. This precipitate was dissolved in dilute hydrochloric acid, ferric chloride was added and the resultant precipitate of ferric phytate was filtered off and thoroughly washed with 2 per cent hydrochloric acid. This washing removed the lower derivatives of phytic acid, leaving essentially pure ferric phytate. The ferric phytate was decomposed with sodium hydroxide, the ferric hydroxide was filtered off and the copper salt was precipitated from the resulting sodium phytate solution with excess copper acetate. In turn the copper salt was decomposed with hydrogen sulfide, the excess hydrogen sulfide was removed by aspiration, and the resultant solution of phytic acid was titrated with sodium hydroxide to pH 8. This solution was used in the solubility studies after suitable dilution.

A solution of sodium phytate derivatives was prepared from a sodium phytate solution by hydrolyzing off half the phosphorus with a phytase-active extract of bran. The so-

lution was then decolorized with charcoal, and the calcium salt was precipitated. This precipitate was dissolved in dilute nitric acid and the major part of the inorganic phosphorus was precipitated as the ammonium molybdiphosphate while the last fraction was removed by isobutyl alcohol extraction in the presence of ammonium molybdate. The calcium salt was reprecipitated, dissolved in acetic acid and the lead salt was then precipitated with lead acetate. This lead salt was then decomposed with hydrogen sulfide, and after the excess hydrogen sulfide had been removed by aspiration the acid solution was titrated to pH 8 with sodium hydroxide. This solution was suitably diluted and used in the solubility studies. It had 2.7 atoms of phosphorus per molecule of phytate derivative.

In the solubility experiments the sodium phytate and phytate derivative solutions were mixed with solutions of the chlorides of the cations under consideration in proportions such that the cation to phosphorus equivalent ratios were 1, 3.75 and 6.25. The concentrations of phosphorus in the final mixtures were 50 and 22.5 p.p.m. for the phytate and phytate derivative forms, respectively. The pH was adjusted with dilute acid or alkali and after the mixtures had stood for 3 to 11 days they were filtered through sintered glass crucibles supporting a layer of

acid-washed, ball-milled quartz sand. The filtrates were then analyzed for phosphorus. The results showed three main features: firstly, that the iron and aluminum salts were very insoluble under acid conditions whereas the calcium and magnesium salts were fully soluble at reactions less than pH 5 and pH 6.5, respectively; secondly, that the phytate derivatives were more soluble than were the phytates especially in the case of the calcium and magnesium salts; and thirdly, that the presence of excess cations decreased the solubilities in the case of the calcium and magnesium salts but with iron and aluminum either caused little change or else increased the solubility at low pH values. This increase in solubility was ascribed to the formation of relatively stable supersaturated solutions and/or the formation of soluble complexes. For the phytates and with the cations in equivalent and excess amounts, respectively, the pH ranges of essentially full insolubility were as follows: for iron from pH 1 to pH 3.5 and from pH 2.5 to pH 8; for aluminum from pH 3 to pH 4 and from pH 3 to pH 9; and for calcium from pH 8 to pH 9 and from pH 6.5 to over pH 10. The magnesium phytate was fully insoluble only with excess magnesium present and at pH values greater than 10. The calcium and magnesium phytate derivatives were always appreciably soluble while the iron and aluminum phytates showed respective minimum

solubilities at pH 2.5 (8 per cent soluble) and pH 4.5 (20 per cent soluble) when equivalent amounts of the cations were present. With excess iron and aluminum present the derivatives were insoluble over the pH ranges from pH 3 to pH 6 and pH 4.5 to pH 8, respectively.

The results for the phytates were compared to those of the corresponding inorganic phosphates found under very similar conditions by a previous worker. The corresponding curves were very similar, the principal contrast being that whereas with equivalent amounts of cations present the inorganic phosphorus was always soluble to an appreciable extent this degree of solubility only applied to the magnesium salt among the phytates. It was concluded that the relatively low solubility of the iron, aluminum and calcium phytates would explain the small availability of phytate phosphorus to plants. It was further concluded that in so far as this insolubility limits the availability and mineralization of phytate phosphorus, agronomic practices designed to maintain the soil reaction in the range pH 6 to pH 6.5 would be desirable.

In the hydrolysis studies, solutions of the chlorides of the cations mentioned above were added to known amounts of sodium phytate and sodium phytate derivatives. The concentrations of phosphorus in the two systems were 46.3 and 27.4 p.p.m., respectively, and the cations were

present in excess except in the case of ferric iron where the cation was present in both equivalent and excess amounts. The reactions were suitably adjusted and a constant amount of a phytase-active extract of bran was added. The mixtures were then incubated at 45° C for 1.75 hours, at the end of which time the increase in inorganic phosphorus was determined. Among the iron and aluminum systems only the ferric phytate derivatives series with the iron present in equivalent amount showed definite hydrolysis. The calcium and magnesium systems were all appreciably hydrolyzed especially in the case of the derivatives. In general the hydrolysis curves followed the solubility curves although there was evidence that as the pH values departed from the optimum for the enzyme (about pH 5.2 to 5.8) the activity of the enzyme was reduced and became a limiting factor with the more soluble salts. It was concluded that in long-term hydrolysis experiments and with the exception of those cases where the pH was either very high or low, the main factor limiting the rate of hydrolysis was the degree of solubility of the phytate.

Prior to investigating the phytase activity of soil it was necessary to develop a method of measuring the phytase activity in soil. An empirical method was developed that consisted of incubating 5 g. of the soil for 20 hours at 45° C with 20 ml. of solution containing 1 g. of potassium citrate and 60 mg. of phytate phosphorus, added

as a solution of sodium phytate, and adjusted to pH 5. Toluene was added to prevent microbial activity. At the end of incubation the soil was filtered and washed with 150 ml. of 4 N hydrochloric acid. The increase in the inorganic phosphorus in this filtrate over that in the filtrate of a sample similarly treated but with the phytate solution replaced by water was taken as a measure of the phytase activity of the soil.

The effects of storage and drying of the soil prior to determining the phytase activity were investigated. It was found that drying greatly reduced the measured activity in three soils of different texture. The decrease in phytase activity was apparently due to the greater adsorption of the phytase by the soil as the moisture content was reduced. One soil, previously incubated with sugar, and which had been dried to varying degrees, was stored in closed bottles and the phytase activity was determined after increasing time intervals. At all moisture levels the activity first increased and then slowly declined. The change was undoubtedly due to microbial synthesis of new enzyme followed by slow decomposition of the enzyme. These results showed that the method was largely dependent on the pretreatment of the samples and accordingly was of most value where the samples were comparable as regards their immediately previous history.

The relationship between overall microbial activity and phytase activity was investigated by measuring the carbon dioxide produced and the phytase activity developed by sterile mixtures of soil and increasing amounts of alfalfa meal when incubated after inoculation from a fresh soil. It was found that the carbon dioxide produced and the level of phytase activity were linearly related, indicating that the higher the microbial activity the higher was the phytase activity in the soil. The results of similar studies using pure bacterial and fungal inocula indicated that phytase production is common to many soil micro-organisms. There was some evidence that fungi might be generally high phytase producers. This was supported by the finding that when soils of different pH values were incubated with energy material, the level of the resulting phytase activity was inversely related to the soil pH. It was concluded that phytase activity is probably present in all field soils and that the level of activity may be greater under more acid conditions.

The findings reported so far indicate that the low solubility of the phytates in acid soils rather than the lack of phytase activity is probably the factor limiting the availability of phytate phosphorus to plants. To obtain more evidence on this point two series of mineralization studies were carried out. In the first series,

the mineralization of sodium phytate added to soils of different pH values was determined and in the second series the mineralization of the native phytate phosphorus in soils of different pH values was determined. The results of the first series of experiments showed that the amount of natural phytase in the soil was sufficient to mineralize a relatively large amount of phytate phosphorus when soluble phytate was present in large amounts. An attempt was made to demonstrate differences in mineralization of added phytate phosphorus due to differences in solubility of the phytates by adding small amounts of sodium phytate to soils of different pH values. This experiment was unsuccessful because the effects of variable pH values could not be controlled and because the increases in inorganic phosphorus that would have been significant were too small to be measured accurately.

In the second series of experiments soils high in organic phosphorus were adjusted to pH values of 5, 6, and 7 and incubated for 15 weeks dry, moist, and moist with starch, sugar and nitrogen added. The soils were then analyzed for phytate phosphorus according to the method described below. Where the soils had been incubated with only water added, there was no decrease in phytate phosphorus irrespective of the soil pH. With the energy material and nitrogen added there was no decrease in phy-

tate phosphorus at pH 5 but appreciable decreases occurred at pH 6 and 7, the greater decrease being found at pH 7. These results may be explained on the basis that at pH 5 the factor limiting mineralization was the low solubility of the soil phytates, whereas at pH 6 and 7 both phytate solubility and phytase activity were limiting factors. However, preliminary evidence was obtained to indicate that soil micro-organisms can synthesize phytates. If further work upholds the present evidence, the explanation of the results obtained in the foregoing experiment must take into account a balance between microbial synthesis and destruction of phytates.

The method developed for the determination of phytate phosphorus in soil was essentially as follows. The organic matter of an 0.5 N sodium hydroxide extract of soil was largely removed by acid precipitation at pH 0.1. The phytate was then precipitated with iron at pH 1.7 to pH 1.8 and the organic matter accompanying this precipitate was destroyed by hypobromite oxidation. This stage was followed by a calcium precipitation under alkaline conditions. The calcium salt was redissolved in dilute acid and the iron precipitation was repeated. Finally, the organic phosphorus in the iron precipitate was determined. The recovery of added phytate phosphorus was complete and it was estimated that for phytate derivatives

it was about 80 to 90 per cent. The recovery of added nucleic acids and other organic phosphates was checked and in all cases was found to be negligible. It was considered that the method is basically sound although still subject to some errors.

In general, the results reported above indicate that soil phytates are relatively stable, especially in acid soils. Soil phytate phosphorus undergoes mineralization, but the extent of mineralization is appreciable only at higher pH values and at high levels of microbial activity. It is probable that under field conditions, where the level of microbial activity is relatively low, phytates supply very little phosphorus to plants during any one growing season.

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